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INVENTOR(s)

LAST NAME	FIRST NAME	M.I.	RESIDENCE (city & either state or foreign country)
Cooper Enright Fioretti	Richard Frederick William	K. M. C.	Baton Rouge, LA Baton Rouge, LA Grapevine, TX

TITLE OF THE INVENTION (280 characters max)

GENE THERAPY FOR ANIMALS

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Respectfully submitted,

SIGNATURE:

*John K. McDonald*

Date: April 26, 2004

TYPED OR PRINTED NAME: John K. McDonald, Ph.D.

Reg. No. 42,860

☐ Additional inventors are being named on separately numbered sheets attached hereto.

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## GENE THERAPY FOR ANIMALS

### FIELD OF THE INVENTION

The present invention relates generally to administration of transposon-based vectors to animals for providing gene therapy to animals. The vectors of the present invention may be directed to specific tissues, organs and cells and produce proteins, peptides and nucleic acids which have a therapeutic effect in the animal.

### BACKGROUND OF THE INVENTION

Improved gene delivery technologies are needed for the treatment of disease in animals. Many diseases and conditions can be treated with gene-delivery technologies, which provide a gene of interest to an animal suffering from the disease or the condition. An example of such disease is Type 1 diabetes. Type 1 diabetes is an autoimmune disease that ultimately results in destruction of the insulin producing  $\beta$ -cells in the pancreas. Although animals with Type 1 diabetes may be treated adequately with insulin injections or insulin pumps, these therapies are only partially effective. In addition, hyper- and hypoglycemia occurs frequently despite intensive home blood glucose monitoring. Finally, careful dietary constraints are needed to maintain an adequate ratio of calories consumed. Development of gene therapies providing delivery of the insulin gene into the pancreas of diabetic animals could overcome many of these problems and result in improved life expectancy and quality of life.

Several of the prior art gene delivery technologies employed viruses that are associated with potentially undesirable side effects and safety concerns. The majority

of current gene-delivery technologies useful for gene therapy rely on virus-based delivery vectors, such as adeno and adeno-associated viruses, retroviruses, and other viruses, which have been attenuated to no longer replicate. (Kay, M.A., et al. 2001. *Nature Medicine* 7:33-40).

5           There are multiple problems associated with the use of viral vectors. First, they are not tissue-specific. In fact, a gene therapy trial using adenovirus was recently halted because the vector was present in a patient's sperm (Gene trial to proceed despite fears that therapy could change child's genetic makeup. *The New York Times*, December 23, 2001). Second, viral vectors are likely to be transiently  
10 incorporated, which necessitates re-treating a patient at specified time intervals. (Kay, M.A., et al. 2001. *Nature Medicine* 7:33-40). Third, there is a concern that a viral-based vector could revert to its virulent form and cause disease. Fourth, viral-based vectors require a dividing cell for stable integration. Fifth, viral-based vectors indiscriminately integrate into various cells, which can result in undesirable germline  
15 integration. Sixth, the required high titers needed to achieve the desired effect have resulted in the death of one patient and they are believed to be responsible for induction of cancer in a separate study. (*Science, News of the Week*, October 4, 2002).

          Accordingly, what is needed is a new method to produce transgenic animals  
20 with stably incorporated genes, in which the vector containing those genes does not cause disease or other unwanted side effects. There is also a need for DNA constructs that would be stably incorporated into the tissues and cells of animals, including cells in the resting state that are not replicating. There is a further recognized need in the art for DNA constructs capable of delivering genes to specific tissues and cells of  
25 animals.

          When incorporating a gene of interest into an animal for the production of a desired protein or when incorporating a gene of interest in an animal for the treatment of a disease, it is often desirable to selectively activate incorporated genes using inducible promoters. These inducible promoters are regulated by substances either  
30 produced or recognized by the transcription control elements within the cell in which the gene is incorporated. In many instances, control of gene expression is desired in transgenic animals so that incorporated genes are selectively activated at desired times and/or under the influence of specific substances. Accordingly, what is needed is a means to selectively activate genes introduced into the genome of cells of a transgenic

animal. This can be taken a step further to cause incorporation to be tissue-specific, which prevents widespread gene incorporation throughout an animal's body. This decreases the amount of DNA needed for a treatment, decreases the chance of incorporation in gametes, and targets gene delivery, incorporation, and expression to  
5 the desired tissue where the gene is needed to function.

## SUMMARY OF THE INVENTION

The present invention addresses the problems described above by providing new, effective and efficient compositions comprising transposon-based vectors for  
10 providing gene therapy to animals. Transgenic animals include all egg-laying animals and milk-producing animals. Transgenic animals further include but are not limited to avians, fish, amphibians, reptiles, insects, and mammals. In another embodiment, the animal is a milk-producing animal, including but not limited to bovine, porcine, ovine and equine animals. In one embodiment, the animal is an avian animal. In  
15 another embodiment, the animal is a mammal. Preferred animals may be pets, domestic animals, exotic animals, zoo animals, wild animals or any other type of animal in need of gene therapy. Animals are made transgenic through administration of a composition comprising a transposon-based vector designed for stable incorporation of a gene of interest for production of a desired protein, peptide or  
20 nucleic acid, together with an acceptable carrier.

The present invention addresses the problems described above by providing methods and compositions for cell and tissue-specific delivery of transposon-based vectors for stable incorporation of an exogenous gene into a specific cell or tissue of an animal. The present invention accomplishes delivery of transposon-based vectors  
25 to a desired cell or tissue for specific incorporation by using cell or tissue-specific transfection reagents. The cell or tissue-specific transfection reagents are engineered by identifying a target receptor specific to the cell or nuclear membrane of the desired cell type. A ligand to the receptor is linked to a transfecting agent, and the resulting ligand-bearing transfection reagent is combined with the transposon-based vector to  
30 provide a composition for specific delivery of a gene of interest to a desired cell or tissue for incorporation and subsequent expression of the gene of interest in the cell or the tissue.

The methods and compositions of the present invention can be used for targeted gene delivery to any cell type possessing a unique receptor. In an

embodiment of the present invention, the transposon-based vectors are designed for tissue or cell-specific gene expression, for example, by placing a selected gene under control of a cell or tissue-specific promoter, which further increases cell or tissue specificity of expression of the selected gene. In one embodiment of the present invention, the vectors are used for gene therapy of animals, wherein the expression of the selected gene has a therapeutic effect on a cell or a tissue in which it is specifically incorporated, expressed, or both. The expression of the selected gene may also have a therapeutic effect on other cells or tissues, particularly when the expressed protein is secreted from the cell and can access other cells. In another embodiment, the vectors are used to integrate a desired gene into specific cells or tissues of an animal for production of biologic agents encoded by the gene of interest.

In another embodiment of the present invention, the gene of interest incorporated into the tissue or cell is expressed and the resulting protein or peptide has regulatory properties affecting a function of the cell or tissue in which it is expressed.

In another embodiment of the present invention, the gene of interest incorporated into the tissue or cell is expressed an inhibitory molecule, such as an RNAi may regulate the overexpression of another substance.

Cell or tissue-specific expression of a gene of interest may be particularly advantageous because of the possible toxic or otherwise potential negative effects of the gene of interest if it were expressed in an undesirable location. Control of expression in the desired cell or tissue is achieved by operably linking the gene of interest to a cell or tissue specific regulator.

In yet another embodiment of the present invention, the expression of the exogenous gene is blocked unless an animal is at a desirable stage in its life cycle. For example, expression of an exogenous gene may not be desirable until an animal reaches puberty. In this embodiment, the expression of the exogenous gene is controlled by a promoter, which is activated specifically at the desired stage in the life of the animal.

In yet another embodiment of the present invention, the expression of the exogenous gene is blocked unless an animal produces a substance. For example, expression of an exogenous gene may not be desirable until an animal begins producing cancer-related molecules. In this embodiment, the expression of the exogenous gene is controlled by a promoter, which is activated specifically by the

cancer-related molecules. Such gene therapy may begin to fight the disease process at very early stages.

5 An additional advantage of the present invention is that a disease or a condition can be treated in a specific organ or tissue of an animal without risk of making other organs or tissues transgenic. This is particularly useful when concerns exist about passing a transgene to the progeny of the transgenic animal, or contaminating the environment with the transgene shed by the animal. The methods and compositions of the present invention are particularly advantageous in applications where germline integration of exogenous DNA is undesirable.

10 The compositions of the present invention may be introduced into an animal through any route of administration that serves to deliver the composition to the desired organs, tissues and cells. Such routes of administration include, but are not limited to, oral and parenteral routes such as intravascular, intravenous, intraarterial, intraperitoneal, anal, intracerebrovascular, intracerebroventricular, cutaneous,  
15 intradermal, subcutaneous, transdermal, into any duct system, into any cavity, intrathecal, or into the respiratory system, the urinary system, the gastrointestinal system, the nervous system, the lymphatic system, the reproductive system and the endocrine system.

The compositions of the present invention may be administered to a  
20 reproductive organ including, but not limited to, an oviduct, an ovary, the testes, seminal vesicle, any accessory organ, or into the duct system of the mammary gland. The compositions of the present invention may be administered to a reproductive organ of an animal through the cloaca. The compositions of the present invention may be directly administered to an organ or can be administered to an artery or vein  
25 leading to the organ. A transfection reagent is optionally added to the composition before administration.

The transposon-based vectors of the present invention include a transposase, operably-linked to a first promoter, and a coding sequence for a protein or peptide of interest operably-linked to a second promoter, wherein the coding sequence for the  
30 protein or peptide of interest and its operably-linked promoter are flanked by transposase insertion sequences recognized by the transposase. The transposon-based vector also includes the following characteristics: a) one or more modified Kozak sequences at the 3' end of the first promoter to enhance expression of the transposase; b) modifications of the codons for the first several N-terminal amino acids of the



transposase, wherein the nucleotide at the third base position of each codon is changed to an A or a T without changing the corresponding amino acid; c) addition of one or more stop codons to enhance the termination of transposase synthesis; and/or, d) addition of an effective polyA sequence operably-linked to the transposase to further enhance expression of the transposase gene. In some embodiments, the effective polyA sequence is an avian optimized polyA sequence.

The present invention also provides for tissue-specific incorporation and/or expression of a gene of interest. Tissue-specific incorporation of a gene of interest may be achieved by placing the transposase gene under the control of a tissue-specific promoter, whereas tissue-specific expression of a gene of interest may be achieved by placing the gene of interest under the control of a tissue-specific promoter. Such tissues include all tissues within the body, for example, connective tissue, muscle, bone, and nervous tissue. In some embodiments, the gene of interest is transcribed under the influence of an ovalbumin, or other oviduct specific, promoter. In other embodiments, promoters may be specific for another organ including but not limited to the liver, brain, breast, and thymus. Tissue specific promoters are known to one of ordinary skill in the art.

The present invention also provides for cell-specific incorporation and/or expression of a gene of interest. Cell-specific incorporation of a gene of interest may be achieved by placing the transposase gene under the control of a cell-specific promoter, whereas tissue-specific expression of a gene of interest may be achieved by placing the gene of interest under the control of a cell-specific promoter. Such promoters may include promoters specific for cells such as neurons, glia, hepatocytes, fibroblasts, chondrocytes, synovial cells, osteoblasts, osteocytes, osteoclasts, muscle cells (including striated, smooth and cardiac muscle cells), lymphocytes, T lymphocytes, B-lymphocytes, thymocytes, germ cells, blast cells, cancerous cells, endocrine cells, white blood cells, pancreatic islet cells, acinar cells, splenocytes, follicular cells, and so on. Cell specific promoters are known to one of ordinary skill in the art.

The present invention advantageously produces a high number of transgenic animals having a gene of interest stably incorporated. In some embodiments wherein the transposon-based vector is administered to the ovary or the testes, these transgenic animals successfully pass the desired gene to their progeny. Accordingly, the present invention can be used to obtain transgenic animals having the gene of interest

incorporated into the germline through transfection of the ovary or testes. These transgenic animals of the present invention produce large amounts of a desired molecule encoded by the transgene. Such germline transmission can produce generations of animals containing the desired gene. Such a gene may be useful in  
5 providing gene therapy to animals known to be susceptible to specific conditions, for example an immune deficiency or arthritis.

Any desired gene may be incorporated into the novel transposon-based vectors of the present invention in order to synthesize a desired molecule in the transgenic animals to provide gene therapy. Proteins, peptides and nucleic acids are preferred  
10 desired molecules to be produced by the transgenic animals of the present invention. In order to provide gene therapy to an animal, the gene desired to produce a desired molecule in the transgenic animal is selected and inserted into the transposon-based vectors of the present invention.

A wide range of recombinant peptides and proteins can be produced in  
15 animals receiving the gene therapy of the present invention. Enzymes, hormones, antibodies, growth factors, serum proteins, commodity proteins, fusion proteins, fusion peptides, biological response modifiers, peptides and designed proteins may all be made through practice of the present invention.

Accordingly, it is an object of the present invention to provide novel  
20 transposon-based vectors useful in providing gene therapy to an animal.

It is another object of the present invention to provide novel transposon-based vectors that encode for the production of desired proteins or peptides in cells or tissues.

It is a further object of the present invention to provide methods for specific  
25 delivery of transposon-based DNA constructs comprising targeting a selected gene to a specific cell or tissue of an animal, comprising complexing DNA constructs containing the selected gene with a transfection reagent adapted for the cell or tissue-specific delivery of the DNA construct to form a composition, and delivering the composition to the cell or the tissue.

30 It is yet another object of the present invention to provide methods for specific expression of transposon-based DNA constructs comprising targeting a selected gene to a specific cell or tissue of an animal, comprising complexing DNA constructs containing the selected gene with a transfection reagent adapted for the cell or tissue-specific delivery of the DNA construct to form a composition, delivering the

composition to the cell or the tissue and expressing of the selected gene in the cell or tissue

It is an object of the present invention to provide gene therapy for generations through germ line administration of a transposon-based vector.

5 It is further an object of the present invention to provide a method to produce transgenic animals through intraovarian or intratesticular administration of a transposon-based vector that are capable of producing transgenic progeny.

Another object of the present invention is to provide gene therapy in animals through administration of a transposon-based vector, wherein the animals produce  
10 desired proteins, peptides or nucleic acids.

Yet another object of the present invention is to provide gene therapy in animals through administration of a transposon-based vector, wherein the animals produce desired proteins or peptides that are recognized by receptors on target cells.

Still another object of the present invention is to provide gene therapy in  
15 animals through administration of a transposon-based vector, wherein the animals produce desired fusion proteins or fusion peptides, a portion of which are recognized by receptors on target cells, in order to deliver the other protein or peptide component of the fusion protein or fusion peptide to the cell to induce a biological response.

Yet another object of the present invention is to provide a method for gene  
20 therapy of animals through administration of transposon-based vectors comprising tissue specific promoters and a gene of interest to facilitate tissue specific incorporation and expression of a gene of interest to produce a desired protein, peptide or nucleic acid.

Another object of the present invention is to provide a method for gene  
25 therapy of animals through administration of transposon-based vectors comprising cell specific promoters and a gene of interest to facilitate cell specific incorporation and expression of a gene of interest to produce a desired protein, peptide or nucleic acid.

Still another object of the present invention is to provide a method for gene  
30 therapy of animals through administration of transposon-based vectors comprising cell specific promoters and a gene of interest to facilitate cell specific incorporation and expression of a gene of interest to produce a desired protein, peptide or nucleic acid, wherein the desired protein, peptide or nucleic acid has a desired biological effect in the animal.

Another object of the present invention is to provide transgenic animals that contain a stably incorporated transgene.

An advantage of the present invention is that transgenic animals are produced with higher efficiencies than observed in the prior art.

5 Another advantage of the present invention is that these transgenic animals possess high copy numbers of the transgene.

Another advantage of the present invention is that the transgenic animals produce large amounts of desired molecules encoded by the transgene.

10 Still another advantage of the present invention is that desired molecules are produced by the transgenic animals much more efficiently and economically than prior art methods, thereby providing a means for efficient gene therapy.

Yet another advantage of the present invention is that the desired proteins and peptides are produced rapidly after making animals transgenic through introduction of the vectors of the present invention.

15 These and other objects, features and advantages of the present invention will become apparent after a review of the following detailed description of the disclosed embodiments and claims.

## BRIEF DESCRIPTION OF THE FIGURES

20 Figure 1 depicts schematically a transposon-based vector containing a transposase operably linked to a first promoter and a gene of interest operably-linked to a second promoter, wherein the gene of interest and its operably-linked promoter are flanked by insertion sequences (IS) recognized by the transposase. "Pro" designates a promoter. In this and subsequent figures, the size of the actual nucleotide  
25 sequence is not necessarily proportionate to the box representing that sequence.

Figure 2 depicts schematically a transposon-based vector for targeting deposition of a polypeptide in an egg white wherein Ov pro is the ovalbumin promoter, Ov protein is the ovalbumin protein and PolyA is a polyadenylation sequence. The TAG sequence includes a spacer sequence, the gp41 hairpin loop from  
30 HIV I and a protease cleavage site.

Figure 3 depicts schematically a transposon-based vector for targeting deposition of a polypeptide in an egg white wherein Ovo pro is the ovomucoid promoter and Ovo SS is the ovomucoid signal sequence. The TAG sequence includes a spacer, the gp41 hairpin loop from HIV I and a protease cleavage site.

Figure 4 depicts schematically a transposon based-vector for expression of an RNAi molecule. "Tet<sub>i</sub> pro" indicates a tetracycline inducible promoter whereas "pro" indicates the pro portion of a prepro sequence as described herein. "Ovgen" indicates approximately 60 base pairs of an ovalbumin gene, "Ovotrans" indicates approximately 60 base pairs of an ovotransferring gene and "Ovomucin" indicates approximately 60 base pairs of an ovomucin gene.

Figure 5 is a picture of an SDS-PAGE gel wherein a pooled fraction of an isolated proinsulin fusion protein was run in lanes 4 and 6. Lanes 1 and 10 of the gel contain molecular weight standards, lanes 2 and 8 contain non-transgenic chicken egg white, and lanes 3, 5, 7 and 9 are blank.

Figure 6 is a schematic of a vector for stable transformation of liver cells for production of insulin.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a new, effective and efficient method of providing gene therapy to animals through administration of a composition comprising a transposon-based vector designed for incorporation of a gene of interest and production of a desired molecule.

#### Definitions

It is to be understood that as used in the specification and in the claims, "a" or "an" can mean one or more, depending upon the context in which it is used. Thus, for example, reference to "a cell" can mean that at least one cell can be utilized.

The term "antibody" is used interchangeably with the term "immunoglobulin" and is defined herein as a protein synthesized by an animal or a cell of the immune system in response to the presence of a foreign substance commonly referred to as an "antigen" or an "immunogen". The term antibody includes fragments of antibodies. Antibodies are characterized by specific affinity to a site on the antigen, wherein the site is referred to an "antigenic determinant" or an "epitope". Antigens can be naturally occurring or artificially engineered. Artificially engineered antigens include, but are not limited to, small molecules, such as small peptides, attached to haptens such as macromolecules, for example proteins, nucleic acids, or polysaccharides. Artificially designed or engineered variants of naturally occurring antibodies and artificially designed or engineered antibodies not occurring in nature

are all included in the current definition. Such variants include conservatively substituted amino acids and other forms of substitution as described in the section concerning proteins and polypeptides.

More particularly, the present invention provides novel transposon-based  
5 vectors and their use for specific incorporation of a gene of interest into a specific cell or a tissue of an animal through the use of a ligand-bearing transfection reagent (LTR). As used herein, the term ligand-bearing transfection reagent (LTR) refers to a composition comprising a transfection reagent linked to a ligand of a receptor specific to the cell or the tissue.

10 The term “gene” is defined herein to include a coding region for a protein, peptide or polypeptide.

The term “transgenic animal” refers to an animal having at least a portion of the transposon-based vector DNA incorporated into its DNA. While a transgenic animal includes an animal wherein the transposon-based vector DNA is incorporated  
15 into the germline DNA, a transgenic animal also includes an animal having DNA in one or more cells that contain a portion of the transposon-based vector DNA for any period of time. In a preferred embodiment, a portion of the transposon-based vector comprises a gene of interest. More preferably, the gene of interest is incorporated into the animal’s DNA for a period of at least a few days, preferably the reproductive life  
20 of the animal, and preferably the life of the animal.

The term “vector” is used interchangeably with the terms “construct”, “DNA construct” and “genetic construct” to denote synthetic nucleotide sequences used for manipulation of genetic material, including but not limited to cloning, subcloning, sequencing, or introduction of exogenous genetic material into cells, tissues or  
25 organisms, such as animals. It is understood by one skilled in the art that vectors may contain synthetic DNA sequences, naturally occurring DNA sequences, or both. The vectors of the present invention are transposon-based vectors as described herein.

When referring to two nucleotide sequences, one being a regulatory sequence, the term “operably-linked” is defined herein to mean that the two sequences are  
30 associated in a manner that allows the regulatory sequence to affect expression of the other nucleotide sequence. It is not required that the operably-linked sequences be directly adjacent to one another with no intervening sequence(s).

The term “regulatory sequence” is defined herein as including promoters, enhancers and other expression control elements such as polyadenylation sequences,

matrix attachment sites, insulator regions for expression of multiple genes on a single construct, ribosome entry/attachment sites, introns that are able to enhance expression, and silencers. Promoters may be cell specific or tissue specific to facilitate expression in a desired target.

5

#### Transposon-Based Vectors

While not wanting to be bound by the following statement, it is believed that the nature of the DNA construct is an important factor in successfully providing gene therapy to animals. The “standard” types of plasmid and viral vectors that have previously been almost universally used for transgenic work in all species have low efficiencies and may constitute a major reason for the low rates of transformation previously observed. The DNA (or RNA) constructs previously used often do not integrate into the host DNA, or integrate only at low frequencies. Other factors may have also played a part, such as poor entry of the vector into target cells. The present invention provides transposon-based vectors that can be administered to an animal that overcome the prior art problems relating to low transgene integration frequencies. Two preferred transposon-based vectors of the present invention in which a transposase, gene of interest and other polynucleotide sequences may be introduced are termed pTnMCS (SEQ ID NO:2) and pTnMod (SEQ ID NO:3).

20 The transposon-based vectors of the present invention produce integration frequencies an order of magnitude greater than has been achieved with previous vectors. More specifically, intratesticular injections performed with a prior art transposon-based vector (described in U.S. Patent No. 5,719,055) resulted in 41% sperm positive roosters whereas intratesticular injections performed with the novel transposon-based vectors of the present invention resulted in 77% sperm positive roosters. Actual frequencies of integration were estimated by either or both comparative strength of the PCR signal from the sperm and histological evaluation of the testes and sperm by quantitative PCR.

25 The transposon-based vectors of the present invention include a transposase gene operably-linked to a first promoter, and a coding sequence for a desired protein or peptide operably-linked to a second promoter, wherein the coding sequence for the desired protein or peptide and its operably-linked promoter are flanked by transposase insertion sequences recognized by the transposase. The transposon-based vector also includes one or more of the following characteristics: a) one or more modified Kozak

sequences comprising ACCATG (SEQ ID NO:1) at the 3' end of the first promoter to enhance expression of the transposase; b) modifications of the codons for the first several N-terminal amino acids of the transposase, wherein the third base of each codon was changed to an A or a T without changing the corresponding amino acid; c) 5 addition of one or more stop codons to enhance the termination of transposase synthesis; and/or, d) addition of an effective polyA sequence operably-linked to the transposase to further enhance expression of the transposase gene. The transposon-based vector may additionally or alternatively include one or more of the following Kozak sequences at the 3' end of any promoter, including the promoter operably- 10 linked to the transposase: ACCATGG (SEQ ID NO:4), AAGATGT (SEQ ID NO:5), ACGATGA (SEQ ID NO:6), AAGATGG (SEQ ID NO:7), GACATGA (SEQ ID NO:8), ACCATGA (SEQ ID NO:9), and ACCATGA (SEQ ID NO:10), ACCATGT (SEQ ID NO:52).

Figure 1 shows a schematic representation of several components of the 15 transposon-based vector. The present invention further includes vectors containing more than one gene of interest, wherein a second or subsequent gene of interest is operably-linked to the second promoter or to a different promoter. It is also to be understood that the transposon-based vectors shown in the Figures are representative of the present invention and that the order of the vector elements may be different 20 than that shown in the Figures, that the elements may be present in various orientations, and that the vectors may contain additional elements not shown in the Figures.

#### Transposases and Insertion Sequences

25 In a further embodiment of the present invention, the transposase found in the transposase-based vector is an altered target site (ATS) transposase and the insertion sequences are those recognized by the ATS transposase. However, the transposase located in the transposase-based vectors is not limited to a modified ATS transposase and can be derived from any transposase. Transposases known in the prior art include 30 those found in AC7, Tn5SEQ1, Tn916, Tn951, Tn1721, Tn 2410, Tn1681, Tn1, Tn2, Tn3, Tn4, Tn5, Tn6, Tn9, Tn10, Tn30, Tn101, Tn903, Tn501, Tn1000 ( $\gamma\delta$ ), Tn1681, Tn2901, AC transposons, Mp transposons, Spm transposons, En transposons, Dotted transposons, Mu transposons, Ds transposons, dSpm transposons and I transposons.



According to the present invention, these transposases and their regulatory sequences are modified for improved functioning as follows: a) the addition one or more modified Kozak sequences comprising ACCATG (SEQ ID NO:1) at the 3' end of the promoter operably-linked to the transposase; b) a change of the codons for the first  
5 several amino acids of the transposase, wherein the third base of each codon was changed to an A or a T without changing the corresponding amino acid; c) the addition of one or more stop codons to enhance the termination of transposase synthesis; and/or, d) the addition of an effective polyA sequence operably-linked to the transposase to further enhance expression of the transposase gene.

10 Although not wanting to be bound by the following statement, it is believed that the modifications of the first several N-terminal codons of the transposase gene increase transcription of the transposase gene, in part, by increasing strand dissociation. It is preferable that between approximately 1 and 20, more preferably 3 and 15, and most preferably between 4 and 12 of the first N-terminal codons of the  
15 transposase are modified such that the third base of each codon is changed to an A or a T without changing the encoded amino acid. In one embodiment, the first ten N-terminal codons of the transposase gene are modified in this manner. It is also preferred that the transposase contain mutations that make it less specific for preferred insertion sites and thus increases the rate of transgene insertion as discussed in U.S.  
20 Patent No. 5,719,055.

In some embodiments, the transposon-based vectors are optimized for expression in a particular host by changing the methylation patterns of the vector DNA. For example, prokaryotic methylation may be reduced by using a methylation deficient organism for production of the transposon-based vector. The transposon-  
25 based vectors may also be methylated to resemble eukaryotic DNA for expression in a eukaryotic host.

Transposases and insertion sequences from other analogous eukaryotic transposon-based vectors that can also be modified and used are, for example, the *Drosophila* P element derived vectors disclosed in U.S. Patent No. 6,291,243; the  
30 *Drosophila* mariner element described in Sherman et al. (1998); or the sleeping beauty transposon. See also Hackett et al. (1999); D. Lampe et al., 1999. Proc. Natl. Acad. Sci. USA, 96:11428-11433; S. Fischer et al., 2001. Proc. Natl. Acad. Sci. USA, 98:6759-6764; L. Zagoraiou et al., 2001. Proc. Natl. Acad. Sci. USA, 98:11474-11478; and D. Berg et al. (Eds.), Mobile DNA, Amer. Soc. Microbiol. (Washington,

D.C., 1989). However, it should be noted that bacterial transposon-based elements are preferred, as there is less likelihood that a eukaryotic transposase in the recipient species will recognize prokaryotic insertion sequences bracketing the transgene.

Many transposases recognize different insertion sequences, and therefore, it is to be understood that a transposase-based vector will contain insertion sequences recognized by the particular transposase also found in the transposase-based vector. In a preferred embodiment of the invention, the insertion sequences have been shortened to about 70 base pairs in length as compared to those found in wild-type transposons that typically contain insertion sequences of well over 100 base pairs.

While the examples provided below incorporate a “cut and insert” Tn10 based vector that is destroyed following the insertion event, the present invention also encompasses the use of a “rolling replication” type transposon-based vector. Use of a rolling replication type transposon allows multiple copies of the transposon/transgene to be made from a single transgene construct and the copies inserted. This type of transposon-based system thereby provides for insertion of multiple copies of a transgene into a single genome. A rolling replication type transposon-based vector may be preferred when the promoter operably-linked to gene of interest is endogenous to the host cell and present in a high copy number or highly expressed. However, use of a rolling replication system may require tight control to limit the insertion events to non-lethal levels. Tn1, Tn2, Tn3, Tn4, Tn5, Tn9, Tn21, Tn501, Tn551, Tn951, Tn1721, Tn2410 and Tn2603 are examples of a rolling replication type transposon, although Tn5 could be both a rolling replication and a cut and insert type transposon.

#### Stop Codons and PolyA Sequences

In one embodiment, the transposon-based vector contains two stop codons operably-linked to the transposase and/or to the gene of interest. In an alternate embodiment, one stop codon of UAA or UGA is operably linked to the transposase and/or to the gene of interest.

As used herein an “effective polyA sequence” refers to either a synthetic or non-synthetic sequence that contains multiple and sequential nucleotides containing an adenine base (an A polynucleotide string) and that increases expression of the gene to which it is operably-linked. A polyA sequence may be operably-linked to any gene in the transposon-based vector including, but not limited to, a transposase gene and a gene of interest. A preferred polyA sequence is optimized for use in the host animal.

In one embodiment, the polyA sequence is optimized for use in an avian species and more specifically, a chicken. An avian optimized polyA sequence generally contains a minimum of 40 base pairs, preferably between approximately 40 and several hundred base pairs, and more preferably approximately 75 base pairs that precede the  
5 A polynucleotide string and thereby separate the stop codon from the A polynucleotide string. In one embodiment of the present invention, the polyA sequence comprises a conalbumin polyA sequence as provided in SEQ ID NO:11 and as taken from GenBank accession # Y00407, base pairs 10651-11058. In another embodiment, the polyA sequence comprises a synthetic polynucleotide sequence  
10 shown in SEQ ID NO:12. In yet another embodiment, the polyA sequence comprises an avian optimized polyA sequence provided in SEQ ID NO:13. A chicken optimized polyA sequence may also have a reduced amount of CT repeats as compared to a synthetic polyA sequence.

It is a surprising discovery of the present invention that such an avian  
15 optimized poly A sequence increases expression of a polynucleotide to which it is operably-linked in an avian as compared to a non-avian optimized polyA sequence. It is to be understood that polyA sequences may be optimized for other animals such as mammals and used in the transposon-based vectors of the present invention to provide gene therapy. Accordingly, the present invention includes methods of or increasing  
20 incorporation of a gene of interest wherein the gene of interest resides in a transposon-based vector containing a transposase gene and wherein the transposase gene is operably linked to an avian optimized polyA sequence. The present invention also includes methods of increasing expression of a gene of interest in an avian that includes administering a gene of interest to the avian, wherein the gene of interest is  
25 operably-linked to an avian optimized polyA sequence. An avian optimized polyA nucleotide string is defined herein as a polynucleotide containing an A polynucleotide string and a minimum of 40 base pairs, preferably between approximately 40 and several hundred base pairs, and more preferably approximately 60 base pairs that precede the A polynucleotide string. The present invention further provides  
30 transposon-based vectors containing a gene of interest or transposase gene operably linked to an avian optimized polyA sequence.

### Promoters and Enhancers

The first promoter operably-linked to the transposase gene and the second promoter operably-linked to the gene of interest can be a constitutive promoter or an  
5 inducible promoter. Constitutive promoters include, but are not limited to, immediate early cytomegalovirus (CMV) promoter, herpes simplex virus 1 (HSV1) immediate early promoter, SV40 promoter, lysozyme promoter, early and late CMV promoters, early and late HSV promoters,  $\beta$ -actin promoter, tubulin promoter, Rous-Sarcoma virus (RSV) promoter, and heat-shock protein (HSP) promoter. Inducible promoters  
10 include tissue-specific promoters, developmentally-regulated promoters and chemically inducible promoters. Examples of tissue-specific promoters include the glucose 6 phosphate (G6P) promoter, vitellogenin promoter, ovalbumin promoter, ovomucoid promoter, conalbumin promoter, ovotransferrin promoter, prolactin promoter, kidney uromodulin promoter, and placental lactogen promoter. In one  
15 embodiment, the vitellogenin promoter includes a polynucleotide sequence of SEQ ID NO:14. The G6P promoter sequence may be deduced from a rat G6P gene untranslated upstream region provided in GenBank accession number U57552.1. Examples of developmentally-regulated promoters include the homeobox promoters and several hormone induced promoters. Examples of chemically inducible  
20 promoters include reproductive hormone induced promoters and antibiotic inducible promoters such as the tetracycline inducible promoter and the zinc-inducible metallothionine promoter.

Other inducible promoter systems include the Lac operator repressor system inducible by IPTG (isopropyl beta-D-thiogalactoside) (Cronin, A. et al. 2001. Genes  
25 and Development, v. 15), ecdysone-based inducible systems (Hoppe, U. C. et al. 2000. Mol. Ther. 1:159-164); estrogen-based inducible systems (Brasemann, S. et al. 1993. Proc. Natl. Acad. Sci. 90:1657-1661); progesterone-based inducible systems using a chimeric regulator, GLVP, which is a hybrid protein consisting of the GAL4 binding domain and the herpes simplex virus transcriptional activation domain, VP16,  
30 and a truncated form of the human progesterone receptor that retains the ability to bind ligand and can be turned on by RU486 (Wang, et al. 1994. Proc. Natl. Acad. Sci. 91:8180-8184); CID-based inducible systems using chemical inducers of dimerization (CIDs) to regulate gene expression, such as a system wherein rapamycin induces

dimerization of the cellular proteins FKBP12 and FRAP (Belshaw, P. J. et al. 1996. *J. Chem. Biol.* 3:731-738; Fan, L. et al. 1999. *Hum. Gene Ther.* 10:2273-2285; Shariat, S.F. et al. 2001. *Cancer Res.* 61:2562-2571; Spencer, D.M. 1996. *Curr. Biol.* 6:839-847). Chemical substances that activate the chemically inducible promoters can be  
5 administered to the animal containing the transgene of interest via any method known to those of skill in the art.

Other examples of cell or tissue-specific and constitutive promoters include but are not limited to smooth-muscle SM22 promoter, including chimeric SM22alpha/telokin promoters (Hoggatt A.M. et al., 2002. *Circ Res.* 91(12):1151-9);  
10 ubiquitin C promoter (*Biochim Biophys Acta*, 2003. Jan. 3;1625(1):52-63); Hsf2 promoter; murine COMP (cartilage oligomeric matrix protein) promoter; early B cell-specific mb-1 promoter (Sigvardsson M., et al., 2002. *Mol. Cell Biol.* 22(24):8539-51); prostate specific antigen (PSA) promoter (Yoshimura I. et al., 2002, *J. Urol.* 168(6):2659-64); exorh promoter and pineal expression-promoting element (Asaoka  
15 Y., et al., 2002. *Proc. Natl. Acad. Sci.* 99(24):15456-61); neural and liver ceramidase gene promoters (Okino N. et al., 2002. *Biochem. Biophys. Res. Commun.* 299(1):160-6); PSP94 gene promoter/enhancer (Gabril M.Y. et al., 2002. *Gene Ther.* 9(23):1589-99); promoter of the human FAT/CD36 gene (Kuriki C., et al., 2002. *Biol. Pharm. Bull.* 25(11):1476-8); VL30 promoter (Staplin W.R. et al., 2002. *Blood*  
20 October 24, 2002); and, IL-10 promoter (Brenner S., et al., 2002. *J. Biol. Chem.* December 18, 2002).

Examples of avian promoters include, but are not limited to, promoters controlling expression of egg white proteins, such as ovalbumin, ovotransferrin (conalbumin), ovomucoid, lysozyme, ovomucin, g2 ovoglobulin, g3 ovoglobulin,  
25 ovoflavoprotein, ovostatin (ovomacroglobin), cystatin, avidin, thiamine-binding protein, glutamyl aminopeptidase minor glycoprotein 1, minor glycoprotein 2; and promoters controlling expression of egg-yolk proteins, such as vitellogenin, very low-density lipoproteins, low density lipoprotein, cobalamin-binding protein, riboflavin-binding protein, biotin-binding protein (Awade, 1996. *Z. Lebensm. Unters. Forsch.*  
30 202:1-14). An advantage of using the vitellogenin promoter is that it is active during the egg-laying stage of an animal's life-cycle, which allows for the production of the protein of interest to be temporally connected to the import of the protein of interest into the egg yolk when the protein of interest is equipped with an appropriate targeting sequence. In some embodiments, the avian promoter is an oviduct-specific

promoter. As used herein, the term "oviduct-specific promoter" includes, but is not limited to, ovalbumin; ovotransferrin (conalbumin); ovomucoid; 01, 02, 03, 04 or 05 avidin; ovomucin; g2 ovoglobulin; g3 ovoglobulin; ovoflavoprotein; and ovostatin (ovomacroglobin) promoters.

5           When germline transformation occurs via intraovarian or intratesticular administration, or when hepatocytes are targeted for incorporation of components of a vector through non-germ line administration, liver-specific promoters may be operably-linked to the gene of interest to achieve liver-specific expression of the transgene. Liver-specific promoters of the present invention include, but are not  
10   limited to, the following promoters, vitellogenin promoter, G6P promoter, cholesterol-7-alpha-hydroxylase (CYP7A) promoter, phenylalanine hydroxylase (PAH) promoter, protein C gene promoter, insulin-like growth factor I (IGF-I) promoter, bilirubin UDP-glucuronosyltransferase promoter, aldolase B promoter, furin promoter, metallothioneine promoter, albumin promoter, and insulin promoter.

15           Also included in the present invention are promoters that can be used to target expression of a protein of interest into the milk of a milk-producing animal including, but not limited to,  $\beta$  lactoglobulin promoter, whey acidic protein promoter, lactalbumin promoter and casein promoter.

          When germline transformation occurs via intraovarian or intratesticular  
20   administration, or when cells of the immune system are targeted through non-germ line administration, immune system-specific promoters may be operably-linked to the gene of interest to achieve immune system-specific expression of the transgene. Accordingly, promoters associated with cells of the immune system may also be used. Acute phase promoters such as interleukin (IL)-1 and IL-2 may be employed.  
25   Promoters for heavy and light chain Ig may also be employed. The promoters of the T cell receptor components CD4 and CD8, B cell promoters and the promoters of CR2 (complement receptor type 2) may also be employed. Immune system promoters are preferably used when the desired protein is an antibody protein.

          Also included in this invention are modified promoters/enhancers wherein  
30   elements of a single promoter are duplicated, modified, or otherwise changed. In one embodiment, steroid hormone-binding domains of the ovalbumin promoter are moved from about -6.5 kb to within approximately the first 1000 base pairs of the gene of interest. Modifying an existing promoter with promoter/enhancer elements not found naturally in the promoter, as well as building an entirely synthetic promoter, or

drawing promoter/enhancer elements from various genes together on a non-natural backbone, are all encompassed by the current invention.

Accordingly, it is to be understood that the promoters contained within the transposon-based vectors of the present invention may be entire promoter sequences or fragments of promoter sequences. For example, in one embodiment, the promoter operably linked to a gene of interest is an approximately 900 base pair fragment of a chicken ovalbumin promoter (SEQ ID NO:15). The constitutive and inducible promoters contained within the transposon-based vectors may also be modified by the addition of one or more modified Kozak sequences of ACCATG (SEQ ID NO:1).

As indicated above, the present invention includes transposon-based vectors containing one or more enhancers. These enhancers may or may not be operably-linked to their native promoter and may be located at any distance from their operably-linked promoter. A promoter operably-linked to an enhancer and a promoter modified to eliminate repressive regulatory effects are referred to herein as an “enhanced promoter.” The enhancers contained within the transposon-based vectors may be enhancers found in birds, such as an ovalbumin enhancer, but are not limited to these types of enhancers. In one embodiment, an approximately 675 base pair enhancer element of an ovalbumin promoter is cloned upstream of an ovalbumin promoter with 300 base pairs of spacer DNA separating the enhancer and promoter. In one embodiment, the enhancer used as a part of the present invention comprises base pairs 1-675 of a chicken ovalbumin enhancer from GenBank accession #S82527.1. The polynucleotide sequence of this enhancer is provided in SEQ ID NO:16.

Also included in some of the transposon-based vectors of the present invention are cap sites and fragments of cap sites. In one embodiment, approximately 50 base pairs of a 5' untranslated region wherein the capsite resides are added on the 3' end of an enhanced promoter or promoter. An exemplary 5' untranslated region is provided in SEQ ID NO:17. A putative cap-site residing in this 5' untranslated region preferably comprises the polynucleotide sequence provided in SEQ ID NO:18.

In one embodiment of the present invention, the first promoter operably-linked to the transposase gene is a constitutive promoter and the second promoter operably-linked to the gene of interest is a tissue-specific promoter. In the second embodiment, use of the first constitutive promoter allows for constitutive activation of the transposase gene and incorporation of the gene of interest into virtually all cell types,

including the germline of the recipient animal. Although the gene of interest is incorporated into the germline generally, the gene of interest may only be expressed in a tissue-specific manner to achieve gene therapy. A transposon-based vector having a constitutive promoter operably-linked to the transposase gene can be administered by any route, and in one embodiment, the vector is administered to an ovary, to an artery leading to the ovary or to a lymphatic system or fluid proximal to the ovary. In another embodiment, the transposon-based vector having a constitutive promoter operably-linked to the transposase gene can be administered to vessels supplying the liver, muscle, brain, lung, kidney, heart or any other desired organ, tissue or cellular target.

It should be noted that cell- or tissue-specific expression as described herein does not require a complete absence of expression in cells or tissues other than the preferred cell or tissue. Instead, "cell-specific" or "tissue-specific" expression refers to a majority of the expression of a particular gene of interest in the preferred cell or tissue, respectively.

When incorporation of the gene of interest into the germline is not preferred, the first promoter operably-linked to the transposase gene can be a tissue-specific promoter. For example, transfection of a transposon-based vector containing a transposase gene operably-linked to an oviduct specific promoter such as the ovalbumin promoter provides for activation of the transposase gene and incorporation of the gene of interest in the cells of the oviduct but not into the germline and other cells generally. In this embodiment, the second promoter operably-linked to the gene of interest can be a constitutive promoter or an inducible promoter. In one embodiment, both the first promoter and the second promoter are an ovalbumin promoter. In embodiments wherein tissue-specific expression or incorporation is desired, it is preferred that the transposon-based vector is administered directly to the tissue of interest, to an artery leading to the organ or tissue of interest or to fluids surrounding the organ or tissue of interest. In one embodiment, the tissue of interest is the oviduct and administration is achieved by direct injection into the oviduct or an artery leading to the oviduct. In another embodiment, the tissue of interest is the liver and administration is achieved by direct injection into the portal vein or hepatic artery. In another embodiment, the tissue of interest is cardiac muscle tissue in the heart and administration is achieved by direct injection into the coronary arteries. In another



embodiment, the tissue of interest is neural tissue and administration is achieved by direct injection into a cerebrovascular or spinovascular artery.

Accordingly, cell specific promoters may be used to enhance transcription in selected tissues. In birds, for example, promoters that are found in cells of the fallopian tube, such as ovalbumin, conalbumin, ovomucoid and/or lysozyme, are used in the vectors to ensure transcription of the gene of interest in the epithelial cells and tubular gland cells of the fallopian tube, leading to synthesis of the desired protein encoded by the gene and deposition into the egg white. In mammals, promoters specific for the epithelial cells of the alveoli of the mammary gland, such as prolactin, insulin, beta lactoglobulin, whey acidic protein, lactalbumin, casein, and/or placental lactogen, are used in the design of vectors used for transfection of these cells for the production of desired proteins for deposition into the milk. In liver cells, the G6P promoter may be employed to drive transcription of the gene of interest for protein production. Proteins made in the liver of birds may be delivered to the egg yolk.

In order to achieve higher or more efficient expression of the transposase gene, the promoter and other regulatory sequences operably-linked to the transposase gene may be those derived from the host. These host specific regulatory sequences can be tissue specific as described above or can be of a constitutive nature. For example, an avian actin promoter and its associated polyA sequence can be operably-linked to a transposase in a transposase-based vector for transfection into an avian. Examples of other host specific promoters that could be operably-linked to the transposase include the myosin and DNA or RNA polymerase promoters.

#### Directing Sequences

In some embodiments of the present invention, the gene of interest is operably-linked to a directing sequence or a sequence that provides proper conformation to the desired protein encoded by the gene of interest. As used herein, the term "directing sequence" refers to both signal sequences and targeting sequences. An egg directing sequence includes, but is not limited to, an ovomucoid signal sequence, an ovalbumin signal sequence, a cecropin pre pro signal sequence, and a vitellogenin targeting sequence. The term "signal sequence" refers to an amino acid sequence, or the polynucleotide sequence that encodes the amino acid sequence, that directs the protein to which it is linked to the endoplasmic reticulum in a eukaryote, and more preferably the translocational pores in the endoplasmic reticulum, or the

plasma membrane in a prokaryote, or mitochondria, such as for the purpose of gene therapy for mitochondrial diseases. Signal and targeting sequences can be used to direct a desired protein into, for example, the bloodstream, when the transposon-based vectors are administered to the liver of an animal.

5           Signal sequences can also be used to direct a desired protein into, for example, a secretory pathway for incorporation into the egg yolk or the egg white, when the transposon-based vectors are administered to a bird or other egg-laying animal. One example of such a transposon-based vector is provided in Figure 3 wherein the gene of interest is operably linked to the ovomucoid signal sequence. The present  
10   invention also includes a gene of interest operably-linked to a second gene containing a signal sequence. An example of such an embodiment is shown in Figure 2 wherein the gene of interest is operably-linked to the ovalbumin gene that contains an ovalbumin signal sequence. Other signal sequences that can be included in the transposon-based vectors include, but are not limited to the ovotransferrin and  
15   lysozyme signal sequences. In one embodiment, the signal sequence is an ovalbumin signal sequence including a sequence shown in SEQ ID NO:19. In another embodiment, the signal sequence is a modified ovalbumin signal sequence including a sequence shown in SEQ ID NO:20 or SEQ ID NO:21.

          As also used herein, the term "targeting sequence" refers to an amino acid  
20   sequence, or the polynucleotide sequence encoding the amino acid sequence, which amino acid sequence is recognized by a receptor located on the exterior of a cell. Binding of the receptor to the targeting sequence results in uptake of the protein or peptide operably-linked to the targeting sequence by the cell. One example of a targeting sequence is a vitellogenin targeting sequence that is recognized by a  
25   vitellogenin receptor (or the low density lipoprotein receptor) on the exterior of an oocyte. In one embodiment, the vitellogenin targeting sequence includes the polynucleotide sequence of SEQ ID NO:22. In another embodiment, the vitellogenin targeting sequence includes all or part of the vitellogenin gene. Other targeting sequences include VLDL and Apo E, which are also capable of binding the  
30   vitellogenin receptor. Since the ApoE protein is not endogenously expressed in birds, its presence may be used advantageously to identify birds carrying the transposon-based vectors of the present invention.

### Genes of Interest Encoding Desired Proteins

A gene of interest selected for stable incorporation is designed to encode any desired protein or peptide or to regulate any cellular response. In some embodiments, the desired proteins or peptides are deposited in an egg or in milk. In other embodiments the desired proteins or peptides may be directed to an axon, to a hepatocyte cell membrane for release into the bloodstream, to the membrane of a beta lymphocyte for release into the circulation. It is to be understood that the present invention encompasses transposon-based vectors containing multiple genes of interest. The multiple genes of interest may each be operably-linked to a separate promoter and other regulatory sequence(s) or may all be operably-linked to the same promoter and other regulatory sequences(s). In one embodiment, multiple gene of interest are linked to a single promoter and other regulatory sequence(s) and each gene of interest is separated by a cleavage site or a pro portion of a signal sequence. A gene of interest may contain modifications of the codons for the first several N-terminal amino acids of the gene of interest, wherein the third base of each codon is changed to an A or a T without changing the corresponding amino acid.

Protein and peptide hormones are a preferred class of proteins in the present invention. Such protein and peptide hormones are synthesized throughout the endocrine system and include, but are not limited to, hypothalamic hormones and hypophysiotropic hormones, anterior, intermediate and posterior pituitary hormones, pancreatic islet hormones, hormones made in the gastrointestinal system, renal hormones, thymic hormones, parathyroid hormones, adrenal cortical and medullary hormones. Specifically, hormones that can be produced using the present invention include, but are not limited to, chorionic gonadotropin, corticotropin, erythropoietin, glucagons, IGF-1, oxytocin, platelet-derived growth factor, calcitonin, follicle-stimulating hormone, luteinizing hormone, thyroid-stimulating hormone, insulin, gonadotropin-releasing hormone and its analogs, vasopressin, octreotide, somatostatin, prolactin, adrenocorticotrophic hormone, antidiuretic hormone, thyrotropin-releasing hormone (TRH), growth hormone-releasing hormone (GHRH), dopamine, melatonin, thyroxin ( $T_4$ ), parathyroid hormone (PTH), glucocorticoids such as cortisol, mineralocorticoids such as aldosterone, androgens such as testosterone, adrenaline (epinephrine), noradrenaline (norepinephrine), estrogens such as estradiol, progesterone, glucagons, calcitrol, calciferol, atrial-natriuretic peptide,

gastrin, secretin, cholecystokinin (CCK), neuropeptide Y, ghrelin, PYY<sub>3-36</sub>, angiotensinogen, thrombopoietin, and leptin. By using appropriate polynucleotide sequences, species-specific hormones may be made by transgenic animals.

In one embodiment of the present invention, the gene of interest is a proinsulin  
5 gene and the desired molecule is insulin. Proinsulin consists of three parts: a C-peptide and two strands of amino acids (the alpha and beta chains) that later become linked together to form the insulin molecule. Figures 2 and 3 are schematics of transposon-based vector constructs containing a proinsulin gene operably-linked to an ovalbumin promoter and ovalbumin protein or an ovomucoid promoter and  
10 ovomucoid signal sequence, respectively. In these embodiments, proinsulin is expressed in the oviduct tubular gland cells and then deposited in the egg white. One example of a proinsulin polynucleotide sequence is shown in SEQ ID NO:23, wherein the C-peptide cleavage site spans from Arg at position 31 to Arg at position 65.

Serum proteins including lipoproteins such as high density lipoprotein (HDL),  
15 HDL-Milano and low density lipoprotein, apolipoprotein, albumin, clotting cascade factors, factor VIII, factor IX, fibrinogen, and globulins are also included in the group of desired proteins of the present invention. Immunoglobulins are one class of desired globulin molecules and include but are not limited to IgG, IgM, IgA, IgD, IgE, IgY, lambda chains, kappa chains and fragments thereof; Fc fragments, and Fab fragments.  
20 Desired antibodies include, but are not limited to, naturally occurring antibodies, animal-specific antibodies, humanized antibodies, and hybrid antibodies. Genes encoding modified versions of naturally occurring antibodies or fragments thereof and genes encoding artificially designed antibodies or fragments thereof may be incorporated into the transposon-based vectors of the present invention. Desired  
25 antibodies also include antibodies with the ability to bind specific ligands, for example, antibodies against proteins associated with cancer-related molecules, such as anti-her 2, or anti-CA125. Accordingly, the present invention encompasses a transposon-based vector containing one or more genes encoding a heavy immunoglobulin (Ig) chain and a light Ig chain. Further, more than one gene  
30 encoding for more than one antibody may be administered in one or more transposon-based vectors of the present invention. In this manner, an egg may contain more than one type of antibody in the egg white, the egg yolk or both. In one embodiment, a transposon-based vector contains a heavy Ig chain and a light Ig chain, both operably linked to a promoter.

Antibodies used as therapeutic reagents include but are not limited to antibodies for use in cancer immunotherapy against specific antigens, or for providing passive immunity to an animal against an infectious disease or a toxic agent. Antibodies may be made by the animal receiving the transposon-based vectors to  
5 facilitate the animal's immune response to a selected antigen. Animals receiving gene therapy to enhance resistance to a disease or to fight an ongoing disease, such as cancer, may receive a transposon-based vector containing genes encoding antibodies that bind to epitopes on cancer cells.

Antibodies that may be made with the practice of the present invention  
10 include, but are not limited to primary antibodies, secondary antibodies, designer antibodies, anti-protein antibodies, anti-peptide antibodies, anti-DNA antibodies, anti-RNA antibodies, anti-hormone antibodies, anti-hypophysiotropic peptides, antibodies against non-natural antigens, anti-anterior pituitary hormone antibodies, anti-posterior pituitary hormone antibodies, anti-venom antibodies, anti-tumor marker antibodies,  
15 antibodies directed against epitopes associated with infectious disease, including, anti-viral, anti-bacterial, anti-protozoal, anti-fungal, anti-parasitic, anti-receptor, anti-lipid, anti-phospholipid, anti-growth factor, anti-cytokine, anti-monokine, anti-idiotypic, and anti-accessory (presentation) protein antibodies. Antibodies made with the present invention, as well as light chains or heavy chains, may also be used to inhibit enzyme  
20 activity.

Antibodies that may be produced using the present invention include, but are not limited to, antibodies made against the following proteins: Bovine  $\gamma$ -Globulin, Serum; Bovine IgG, Plasma; Chicken  $\gamma$ -Globulin, Serum; Human  $\gamma$ -Globulin, Serum; Human IgA, Plasma; Human IgA<sub>1</sub>, Myeloma; Human IgA<sub>2</sub>, Myeloma; Human IgA<sub>2</sub>,  
25 Plasma; Human IgD, Plasma; Human IgE, Myeloma; Human IgG, Plasma; Human IgG, Fab Fragment, Plasma; Human IgG, F(ab')<sub>2</sub> Fragment, Plasma; Human IgG, Fc Fragment, Plasma; Human IgG<sub>1</sub>, Myeloma; Human IgG<sub>2</sub>, Myeloma; Human IgG<sub>3</sub>, Myeloma; Human IgG<sub>4</sub>, Myeloma; Human IgM, Myeloma; Human IgM, Plasma; Human Immunoglobulin, Light Chain  $\kappa$ , Urine; Human Immunoglobulin, Light  
30 Chains  $\kappa$  and  $\lambda$ , Plasma; Mouse  $\gamma$ -Globulin, Serum; Mouse IgG, Serum; Mouse IgM, Myeloma; Rabbit  $\gamma$ -Globulin, Serum; Rabbit IgG, Plasma; and Rat  $\gamma$ -Globulin, Serum. In one embodiment, the transposon-based vector comprises the coding sequence of light and heavy chains of a murine monoclonal antibody that shows

specificity for human seminoprotein (GenBank Accession numbers AY129006 and AY129304 for the light and heavy chains, respectively).

A further non-limiting list of antibodies that recognize other antibodies is as follows: Anti-Chicken IgG, heavy (H) & light (L) Chain Specific (Sheep); Anti-Goat  
5  $\gamma$ -Globulin (Donkey); Anti-Goat IgG, Fc Fragment Specific (Rabbit); Anti-Guinea Pig  $\gamma$ -Globulin (Goat); Anti-Human Ig, Light Chain, Type  $\kappa$  Specific; Anti-Human Ig, Light Chain, Type  $\lambda$  Specific; Anti-Human IgA,  $\alpha$ -Chain Specific (Goat); Anti-Human IgA, Fab Fragment Specific; Anti-Human IgA, Fc Fragment Specific; Anti-Human IgA, Secretory; Anti-Human IgE,  $\epsilon$ -Chain Specific (Goat); Anti-Human IgE,  
10 Fc Fragment Specific; Anti-Human IgG, Fc Fragment Specific (Goat); Anti-Human IgG,  $\gamma$ -Chain Specific (Goat); Anti-Human IgG, Fc Fragment Specific; Anti-Human IgG, Fd Fragment Specific; Anti-Human IgG, H & L Chain Specific (Goat); Anti-Human IgG<sub>1</sub>, Fc Fragment Specific; Anti-Human IgG<sub>2</sub>, Fc Fragment Specific; Anti-Human IgG<sub>2</sub>, Fd Fragment Specific; Anti-Human IgG<sub>3</sub>, Hinge Specific; Anti-Human  
15 IgG<sub>4</sub>, Fc Fragment Specific; Anti-Human IgM, Fc Fragment Specific; Anti-Human IgM,  $\mu$ -Chain Specific; Anti-Mouse IgE,  $\epsilon$ -Chain Specific; Anti-Mouse  $\gamma$ -Globulin (Goat); Anti-Mouse IgG,  $\gamma$ -Chain Specific (Goat); Anti-Mouse IgG,  $\gamma$ -Chain Specific (Goat) F(ab')<sub>2</sub> Fragment; Anti-Mouse IgG, H & L Chain Specific (Goat); Anti-Mouse IgM,  $\mu$ -Chain Specific (Goat); Anti-Mouse IgM, H & L Chain Specific (Goat); Anti-  
20 Rabbit  $\gamma$ -Globulin (Goat); Anti-Rabbit IgG, Fc Fragment Specific (Goat); Anti-Rabbit IgG, H & L Chain Specific (Goat); Anti-Rat  $\gamma$ -Globulin (Goat); Anti-Rat IgG, H & L Chain Specific; Anti-Rhesus Monkey  $\gamma$ -Globulin (Goat); and, Anti-Sheep IgG, H & L Chain Specific.

Another non-limiting list of the antibodies that may be produced using the  
25 present invention is provided in product catalogs of companies such as Phoenix Pharmaceuticals, Inc. ([www.phoenixpeptide.com](http://www.phoenixpeptide.com); 530 Harbor Boulevard, Belmont, CA), Peninsula Labs (San Carlos CA), SIGMA (St. Louis, MO [www.sigma-aldrich.com](http://www.sigma-aldrich.com)), Cappel ICN (Irvine, California, [www.icnbiomed.com](http://www.icnbiomed.com)), and Calbiochem (La Jolla, California, [www.calbiochem.com](http://www.calbiochem.com)), which are all incorporated herein by  
30 reference in their entirety. The polynucleotide sequences encoding these antibodies may be obtained from the scientific literature, from patents, and from databases such as GenBank. Alternatively, one of ordinary skill in the art may design the polynucleotide sequence to be incorporated into the genome by choosing the codons that encode for each amino acid in the desired antibody. Antibodies made by the

transgenic animals of the present invention include antibodies that may be used as therapeutic reagents, for example in cancer immunotherapy against specific antigens. Some of these antibodies include, but are not limited to, antibodies which bind the following ligands: adrenomedulin, amylin, calcitonin, amyloid, calcitonin gene-related peptide, cholecystokinin, gastrin, gastric inhibitory peptide, gastrin releasing peptide, interleukin, interferon, cortistatin, somatostatin, endothelin, sarafotoxin, glucagon, glucagon-like peptide, insulin, atrial natriuretic peptide, BNP, CNP, neurokinin, substance P, leptin, neuropeptide Y, melanin concentrating hormone, melanocyte stimulating hormone, orphanin, endorphin, dynorphin, enkephalin, 10 enkephalin, leumorphin, peptide F, PACAP, PACAP-related peptide, parathyroid hormone, urocortin, corticotrophin releasing hormone, PHM, PHI, vasoactive intestinal polypeptide, secretin, ACTH, angiotensin, angiostatin, bombesin, endostatin, bradykinin, FMRF amide, galanin, gonadotropin releasing hormone (GnRH) associated peptide, GnRH, growth hormone releasing hormone, inhibin, 15 granulocyte-macrophage colony stimulating factor (GM-CSF), motilin, neurotensin, oxytocin, vasopressin, osteocalcin, pancreastatin, pancreatic polypeptide, peptide YY, proopiomelanocortin, transforming growth factor, vascular endothelial growth factor, vesicular monoamine transporter, vesicular acetylcholine transporter, ghrelin, NPW, NPB, C3d, prokineticin, thyroid stimulating hormone, luteinizing hormone, follicle stimulating hormone, prolactin, growth hormone, beta-lipotropin, melatonin, kallikriens, kinins, prostaglandins, erythropoietin, p146 (SEQ ID NO:24 amino acid sequence, SEQ ID NO:25, nucleotide sequence), estrogen, testosterone, corticosteroids, mineralocorticoids, thyroid hormone, thymic hormones, connective tissue proteins, nuclear proteins, actin, avidin, activin, agrin, albumin, and 20 prohormones, propeptides, splice variants, fragments and analogs thereof.

The following is yet another non-limiting list of antibodies that can be produced by the methods of present invention: abciximab (ReoPro), abciximab anti-platelet aggregation monoclonal antibody, anti-CD11a (hu1124), anti-CD18 antibody, anti-CD20 antibody, anti-cytomegalovirus (CMV) antibody, anti-digoxin antibody, 30 anti-hepatitis B antibody, anti-HER-2 antibody, anti-idiotypic antibody to GD3 glycolipid, anti-IgE antibody, anti-IL-2R antibody, antimetastatic cancer antibody (mAb 17-1A), anti-rabies antibody, anti-respiratory syncytial virus (RSV) antibody, anti-Rh antibody, anti-TCR, anti-TNF antibody, anti-VEGF antibody and fab fragment thereof, rattlesnake venom antibody, black widow spider venom antibody,

coral snake venom antibody, antibody against very late antigen-4 (VLA-4), C225 humanized antibody to EGF receptor, chimeric (human & mouse) antibody against TNF $\alpha$ , antibody directed against GPIIb/IIIa receptor on human platelets, gamma globulin, anti-hepatitis B immunoglobulin, human anti-D immunoglobulin, human antibodies against *S aureus*, human tetanus immunoglobulin, humanized antibody against the epidermal growth receptor-2, humanized antibody against the  $\alpha$  subunit of the interleukin-2 receptor, humanized antibody CTLA4IG, humanized antibody to the IL-2 R  $\alpha$ -chain, humanized anti-CD40-ligand monoclonal antibody (5c8), humanized mAb against the epidermal growth receptor-2, humanized mAb to rous sarcoma virus, humanized recombinant antibody (IgG1k) against respiratory syncytial virus (RSV), lymphocyte immunoglobulin (anti-thymocyte antibody), lymphocyte immunoglobulin, mAb against factor VII, MDX-210 bi-specific antibody against HER-2, MDX-22, MDX-220 bi-specific antibody against TAG-72 on tumors, MDX-33 antibody to Fc $\gamma$ R1 receptor, MDX-447 bi-specific antibody against EGF receptor, MDX-447 bispecific humanized antibody to EGF receptor, MDX-RA immunotoxin (ricin A linked) antibody, Medi-507 antibody (humanized form of BTI-322) against CD2 receptor on T-cells, monoclonal antibody LDP-02, muromonab-CD3(OKT3) antibody, OKT3 ("muromomab-CD3") antibody, PRO 542 antibody, ReoPro ("abciximab") antibody, and TNF-IgG fusion protein. It is to be understood that wherever the term "humanized" appears in the present patent application with regard to an antibody or molecule, that an antibody or molecule may be designed to be specific for any animal using selected polynucleotide sequences in the gene of interest included in the transposon-based vectors.

The antibodies prepared using the methods of the present invention may also be designed to possess specific labels that may be detected through means known to one of ordinary skill in the art so that their location and distribution can be assessed following gene therapy and expression of the antibodies. The antibodies may also be designed to possess specific sequences useful for purification through means known to one of ordinary skill in the art. Specialty antibodies designed for binding specific antigens may also be made in transgenic animals using the transposon-based vectors of the present invention.

Production of a monoclonal antibody using the transposon-based vectors of the present invention can be accomplished in a variety of ways. In one embodiment, two vectors may be constructed: one that encodes the light chain, and a second vector



that encodes the heavy chain of the monoclonal antibody. These vectors may then be incorporated into the genome of the target animal by methods disclosed herein. In an alternative embodiment, the sequences encoding light and heavy chains of a monoclonal antibody may be included on a single DNA construct. For example, the coding sequence of light and heavy chains of a murine monoclonal antibody that show specificity for human seminoprotein can be expressed using transposon-based constructs of the present invention (GenBank Accession numbers AY129006 and AY129304 for the light and heavy chains, respectively).

Further included in the present invention are proteins and peptides synthesized by the immune system including those synthesized by the thymus, lymph nodes, spleen, and the gastrointestinal associated lymph tissues (GALT) system. The immune system proteins and peptides proteins that can be made in transgenic animals using the transposon-based vectors of the present invention include, but are not limited to, alpha-interferon, beta-interferon, gamma-interferon, alpha-interferon A, alpha-interferon 1, G-CSF, GM-CSF, interleukin-1 (IL-1), IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, TNF- $\alpha$ , and TNF- $\beta$ . Other cytokines included in the present invention include cardiotrophin, stromal cell derived factor, macrophage derived chemokine (MDC), melanoma growth stimulatory activity (MGSA), macrophage inflammatory proteins 1 alpha (MIP-1 alpha), 2, 3 alpha, 3 beta, 4 and 5.

Lytic peptides such as p146 are also included in the desired molecules of the present invention. In one embodiment, the p146 peptide comprises an amino acid sequence of SEQ ID NO:24. The present invention also encompasses a transposon-based vector comprising a p146 nucleic acid comprising a polynucleotide sequence of SEQ ID NO:25.

Enzymes are another class of proteins that may be made through gene therapy of the transposon-based vectors of the present invention. Such enzymes include but are not limited to adenosine deaminase, alpha-galactosidase, cellulase, collagenase, dnaseI, hyaluronidase, lactase, L-asparaginase, pancreatin, papain, streptokinase B, subtilisin, superoxide dismutase, thrombin, trypsin, urokinase, fibrinolysin, glucocerebrosidase and plasminogen activator. In some embodiments wherein the enzyme could have deleterious effects, additional amino acids and a protease cleavage site are added to the carboxy end of the enzyme of interest in order to prevent

expression of a functional enzyme. Subsequent digestion of the enzyme with a protease results in activation of the enzyme.

Extracellular matrix proteins are one class of desired proteins that may be made through the gene therapy of the present invention. Examples include but are not limited to collagen, fibrin, elastin, laminin, and fibronectin and subtypes thereof. Animals receiving gene therapy for conditions such as arthritis or clotting disorders may make some of these matrix proteins. Gene therapy may be administered to stimulate formation of cartilage, such as articular cartilage, or for deposition of new bone. Intracellular proteins and structural proteins are other classes of desired proteins in the present invention.

Growth factors are another desired class of proteins that may be made through the gene therapy of the present invention and include, but are not limited to, transforming growth factor- $\alpha$  ("TGF- $\alpha$ "), transforming growth factor- $\beta$  (TGF- $\beta$ ), platelet-derived growth factors (PDGF), fibroblast growth factors (FGF), including FGF acidic isoforms 1 and 2, FGF basic form 2 and FGF 4, 8, 9 and 10, nerve growth factors (NGF) including NGF 2.5s, NGF 7.0s and beta NGF and neurotrophins, brain derived neurotrophic factor, cartilage derived factor, growth factors for stimulation of the production of red blood cells, growth factors for stimulation of the production of white blood cells, bone growth factors (BGF), basic fibroblast growth factor, vascular endothelial growth factor (VEGF), granulocyte colony stimulating factor (G-CSF), insulin like growth factor (IGF) I and II, hepatocyte growth factor, glial neurotrophic growth factor (GDNF), stem cell factor (SCF), keratinocyte growth factor (KGF), transforming growth factors (TGF), including TGFs alpha, beta, beta1, beta2, beta3, skeletal growth factor, bone matrix derived growth factors, bone derived growth factors, erythropoietin (EPO) and mixtures thereof.

Another desired class of proteins that may be made may be made through the gene therapy of the present invention include, but are not limited to, leptin, leukemia inhibitory factor (LIF), tumor necrosis factor alpha and beta, ENBREL, angiostatin, endostatin, thrombospondin, osteogenic protein-1, bone morphogenetic proteins 2 and 7, osteonectin, somatomedin-like peptide, and osteocalcin.

Yet another desired class of proteins are blood proteins or clotting cascade protein including albumin, Prekallikrein, High molecular weight kininogen (HMWK) (contact activation cofactor; Fitzgerald, Flaujeac Williams factor), Factor I

(Fibrinogen), Factor II (prothrombin), Factor III (Tissue Factor), Factor IV (calcium), Factor V (proaccelerin, labile factor, accelerator (Ac-) globulin), Factor VI (Va) (accelerin), Factor VII (proconvertin), serum prothrombin conversion accelerator (SPCA), cothromboplastin), Factor VIII (antihemophilic factor A, antihemophilic  
5 globulin (AHG)), Factor IX (Christmas Factor, antihemophilic factor B, plasma thromboplastin component (PTC)), Factor X (Stuart-Prower Factor), Factor XI (Plasma thromboplastin antecedent (PTA)), Factor XII (Hageman Factor), Factor XIII (rotransglutaminase, fibrin stabilizing factor (FSF), fibrinoligase), von Willebrand factor, Protein C, Protein S, Thrombomodulin, Antithrombin III.

10 A non-limiting list of the peptides and proteins that may be made may be made through the use of the gene therapy methods of the present invention is provided in product catalogs of companies such as Phoenix Pharmaceuticals, Inc. ([www.phoenixpeptide.com](http://www.phoenixpeptide.com); 530 Harbor Boulevard, Belmont, CA), Peninsula Labs (San Carlos CA), SIGMA, (St.Louis, MO [www.sigma-aldrich.com](http://www.sigma-aldrich.com)), Cappel ICN  
15 (Irvine, California, [www.icnbiomed.com](http://www.icnbiomed.com)), and Calbiochem (La Jolla, California, [www.calbiochem.com](http://www.calbiochem.com)). The polynucleotide sequences encoding these proteins and peptides of interest may be obtained from the scientific literature, from patents, and from databases such as GenBank. Alternatively, one of ordinary skill in the art may design the polynucleotide sequence to be incorporated into the genome by choosing  
20 the codons that encode for each amino acid in the desired protein or peptide.

Some of these desired proteins or peptides that may be made through the use of the gene therapy methods of the present invention include but are not limited to the following: adrenomedulin, amylin, calcitonin, amyloid, calcitonin gene-related peptide, cholecystokinin, gastrin, gastric inhibitory peptide, gastrin releasing peptide,  
25 interleukin, interferon, cortistatin, somatostatin, endothelin, sarafotoxin, glucagon, glucagon-like peptide, insulin, atrial natriuretic peptide, BNP, CNP, neurokinin, substance P, leptin, neuropeptide Y, melanin concentrating hormone, melanocyte stimulating hormone, orphanin, endorphin, dynorphin, enkephalin, leumorphin, peptide F, PACAP, PACAP-related peptide, parathyroid hormone, urocortin,  
30 corticotrophin releasing hormone, PHM, PHI, vasoactive intestinal polypeptide, secretin, ACTH, angiotensin, angiotatin, bombesin, endostatin, bradykinin, FMRF amide, galanin, gonadotropin releasing hormone (GnRH) associated peptide, GnRH, growth hormone releasing hormone, inhibin, granulocyte-macrophage colony stimulating factor (GM-CSF), motilin, neurotensin, oxytocin, vasopressin,

osteocalcin, pancreastatin, pancreatic polypeptide, peptide YY, proopiomelanocortin, transforming growth factor, vascular endothelial growth factor, vesicular monoamine transporter, vesicular acetylcholine transporter, ghrelin, NPW, NPB, C3d, prokineticin, thyroid stimulating hormone, luteinizing hormone, follicle stimulating hormone, prolactin, growth hormone, beta-lipotropin, melatonin, kallikriens, kinins, prostaglandins, erythropoietin, p146 (SEQ ID NO:24, amino acid sequence, SEQ ID NO:25, nucleotide sequence), thymic hormones, connective tissue proteins, nuclear proteins, actin, avidin, activin, agrin, albumin, apolipoproteins, apolipoprotein A, apolipoprotein B, and prohormones, propeptides, splice variants, fragments and analogs thereof.

Other desired proteins that may be made by the transgenic animals receiving gene therapy according to the present invention include bacitracin, polymixin b, vancomycin, cyclosporine, anti-RSV antibody, alpha-1 antitrypsin (AAT), anti-cytomegalovirus antibody, anti-hepatitis antibody, anti-inhibitor coagulant complex, anti-rabies antibody, anti-Rh(D) antibody, adenosine deaminase, anti-digoxin antibody, antivenin crotalidae (rattlesnake venom antibody), antivenin latrodectus (black widow spider venom antibody), antivenin micrurus (coral snake venom antibody), aprotinin, corticotropin (ACTH), diphtheria antitoxin, lymphocyte immune globulin (anti-thymocyte antibody), protamine, thyrotropin, capreomycin,  $\alpha$ -galactosidase, gramicidin, streptokinase, tetanus toxoid, tyrothricin, IGF-1, proteins of varicella vaccine, anti-TNF antibody, anti-IL-2r antibody, anti-HER-2 antibody, OKT3 ("muromonab-CD3") antibody, TNF-IgG fusion protein, ReoPro ("abciximab") antibody, ACTH fragment 1-24, desmopressin, gonadotropin-releasing hormone, histrelin, leuprolide, lypressin, nafarelin, peptide that binds GPIIb/GPIIIa on platelets (integrilin), goserelin, capreomycin, colistin, anti-respiratory syncytial virus, lymphocyte immune globulin (Thymoglovin, Atgam), panorex, alpha-antitrypsin, botulinin, lung surfactant protein, tumor necrosis receptor-IgG fusion protein (enbrel), gonadorelin, proteins of influenza vaccine, proteins of rotavirus vaccine, proteins of haemophilus b conjugate vaccine, proteins of poliovirus vaccine, proteins of pneumococcal conjugate vaccine, proteins of meningococcal C vaccine, proteins of influenza vaccine, megakaryocyte growth and development factor (MGDF), neuroimmunophilin ligand-A (NIL-A), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), leptin (native), leptin B, leptin C,

IL-1RA (interleukin-1RA), R-568, novel erythropoiesis-stimulating protein (NESP),  
 humanized mAb to rous sarcoma virus (MEDI-493), glutamyl-tryptophan dipeptide  
 IM862, LFA-3TIP immunosuppressive, humanized anti-CD40-ligand monoclonal  
 antibody (5c8), gelsonin enzyme, tissue factor pathway inhibitor (TFPI), proteins of  
 5 meningitis B vaccine, antimetastatic cancer antibody (mAb 17-1A), chimeric (human  
 & mouse) mAb against TNF $\alpha$ , mAb against factor VII, relaxin, capreomycin,  
 glycopeptide (LY333328), recombinant human activated protein C (rhAPC),  
 humanized mAb against the epidermal growth receptor-2, altepase, anti-CD20  
 antigen, C2B8 antibody, insulin-like growth factor-1, atrial natriuretic peptide  
 10 (anaritide), tenectapase, anti-CD11a antibody (hu 1124), anti-CD18 antibody, mAb  
 LDP-02, anti-VEGF antibody, fab fragment of anti-VEGF Ab, APO2 ligand (tumor  
 necrosis factor-related apoptosis-inducing ligand), rTGF- $\beta$  (transforming growth  
 factor- $\beta$ ), alpha-antitrypsin, ananain (a pineapple enzyme), humanized mAb  
 CTLA4IG, PRO 542 (mAb), D2E7 (mAb), calf intestine alkaline phosphatase,  $\alpha$ -L-  
 15 iduronidase,  $\alpha$ -L-galactosidase (humanglutamic acid decarboxylase, acid  
 sphingomyelinase, bone morphogenetic protein-2 (rhBMP-2), proteins of HIV  
 vaccine, T cell receptor (TCR) peptide vaccine, TCR peptides, V beta 3 and V beta  
 13.1. (IR502), (IR501), BI 1050/1272 mAb against very late antigen-4 (VLA-4),  
 C225 humanized mAb to EGF receptor, anti-idiotypic antibody to GD3 glycolipid,  
 20 antibacterial peptide against *H. pylori*, MDX-447 bispecific humanized mAb to EGF  
 receptor, anti-cytomegalovirus (CMV), Medi-491 B19 parvovirus vaccine, humanized  
 recombinant mAb (IgG1k) against respiratory syncytial virus (RSV), urinary tract  
 infection vaccine (against "pili" on *Escherichia coli* strains), proteins of lyme disease  
 vaccine against *B. burgdorferi* protein (DbpA), proteins of Medi-501 human  
 25 papilloma virus-11 vaccine (HPV), *Streptococcus pneumoniae* vaccine, Medi-507  
 mAb (humanized form of BTI-322) against CD2 receptor on T-cells, MDX-33 mAb  
 to Fc $\gamma$ R1 receptor, MDX-RA immunotoxin (ricin A linked) mAb, MDX-210 bi-  
 specific mAb against HER-2, MDX-447 bi-specific mAb against EGF receptor,  
 MDX-22, MDX-220 bi-specific mAb against TAG-72 on tumors, colony-stimulating  
 30 factor (CSF) (molgramostim), humanized mAb to the IL-2 R  $\alpha$ -chain (basiliximab),  
 mAb to IgE (IGE 025A), myelin basic protein-altered peptide (MSP771A),  
 humanized mAb against the epidermal growth receptor-2, humanized mAb against the  
 $\alpha$  subunit of the interleukin-2 receptor, low molecular weight heparin, anti-  
 hemophillic factor, and bactericidal/permeability-increasing protein (r-BPI).

The peptides and proteins made by animals receiving gene therapy using the present invention may be labeled using labels and techniques known to one of ordinary skill in the art. Some of these labels are described in the "Handbook of Fluorescent Probes and Research Products", ninth edition, Richard P. Haugland (ed) Molecular Probes, Inc. Eugene, OR), which is incorporated herein in its entirety. Some of these labels may be genetically engineered into the polynucleotide sequence for the expression of the selected protein or peptide. The peptides and proteins may also have label-incorporation "handles" incorporated to allow labeling of an otherwise difficult or impossible to label protein.

It is to be understood that the various classes of desired peptides and proteins, as well as specific peptides and proteins described in this section may be modified as described below by inserting selected codons for desired amino acid substitutions into the gene incorporated into the transgenic animal.

The present invention may also be used to produce desired molecules other than proteins and peptides including, but not limited to, lipoproteins such as high density lipoprotein (HDL), HDL-Milano, and low density lipoprotein, lipids, carbohydrates, siRNA and ribozymes. In these embodiments, a gene of interest encodes a nucleic acid molecule or a protein that directs production of the desired molecule.

The present invention further encompasses gene therapy to produce inhibitory molecules to inhibit endogenous (i.e., non-vector) protein production. Such therapy may be used to inhibit a gene that is over expressed. These inhibitory molecules include antisense nucleic acids, siRNA, polynucleotide strands that affect cellular function and inhibitory proteins. In one embodiment, the endogenous protein whose expression is inhibited is an egg white protein including, but not limited to ovalbumin, ovotransferrin, and ovomucin. In one embodiment, a transposon-based vector containing an ovalbumin DNA sequence, that upon transcription forms a double stranded RNA molecule, is transfected into an animal, such as a bird, and the bird's production of endogenous ovalbumin protein is reduced by the interference RNA mechanism (RNAi). In other embodiments, a transposon-based vector encodes an inhibitory RNA molecule that inhibits the expression of more than one egg white protein. One exemplary construct is provided in Figure 4 wherein "Ovgen" indicates approximately 60 base pairs of an ovalbumin gene, "Ovotrans" indicates approximately 60 base pairs of an ovotransferrin gene and "Ovomucin" indicates

approximately 60 base pairs of an ovomucin gene. These ovalbumin, ovotransferrin and ovomucin can be from any avian species, and in some embodiments, are from a chicken or quail. The term "pro" indicates the pro portion of a prepro sequence. One exemplary prepro sequence is that of cecropin and comprising base pairs 563-733 of the Cecropin cap site and Prepro provided in Genbank accession number X07404. Additional cecropin prepro and pro sequences are provided in SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, and SEQ ID NO:51. Additionally, inducible knockouts or knockdowns of the endogenous protein may be created to achieve a reduction or inhibition of endogenous protein production. The approach may be used for inhibition of any selected endogenous protein in animals receiving gene therapy.

#### Modified Desired Proteins and Peptides Made by Animals Receiving Gene Therapy

"Proteins", "peptides," "polypeptides" and "oligopeptides" are chains of amino acids (typically L-amino acids) whose alpha carbons are linked through peptide bonds formed by a condensation reaction between the carboxyl group of the alpha carbon of one amino acid and the amino group of the alpha carbon of another amino acid. The terminal amino acid at one end of the chain (i.e., the amino terminal) has a free amino group, while the terminal amino acid at the other end of the chain (i.e., the carboxy terminal) has a free carboxyl group. As such, the term "amino terminus" (abbreviated N-terminus) refers to the free alpha-amino group on the amino acid at the amino terminal of the protein, or to the alpha-amino group (imino group when participating in a peptide bond) of an amino acid at any other location within the protein. Similarly, the term "carboxy terminus" (abbreviated C-terminus) refers to the free carboxyl group on the amino acid at the carboxy terminus of a protein, or to the carboxyl group of an amino acid at any other location within the protein.

Typically, the amino acids making up a protein are numbered in order, starting at the amino terminal and increasing in the direction toward the carboxy terminal of the protein. Thus, when one amino acid is said to "follow" another, that amino acid is positioned closer to the carboxy terminal of the protein than the preceding amino acid.

The term "residue" is used herein to refer to an amino acid (D or L) or an amino acid mimetic that is incorporated into a protein by an amide bond. As such, the amino acid may be a naturally occurring amino acid or, unless otherwise limited, may encompass known analogs of natural amino acids that function in a manner similar to the naturally occurring amino acids (i.e., amino acid mimetics). Moreover, an amide

bond mimetic includes peptide backbone modifications well known to those skilled in the art.

Furthermore, one of skill will recognize that, as mentioned above, individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids (typically less than about 5%, more typically less than about 1%) in an encoded sequence are conservatively modified variations where the alterations result in the substitution of an amino acid with a chemically similar amino acid. Such substitutions may be engineered by selecting the desired nucleotides for insertion into the gene of interest in animals receiving gene therapy.

Conservative substitution tables providing functionally similar amino acids are well known in the art. The following six groups each contain amino acids that are conservative substitutions for one another:

- 1) Alanine (A), Serine (S), Threonine (T);
- 2) Aspartic acid (D), Glutamic acid (E);
- 3) Asparagine (N), Glutamine (Q);
- 4) Arginine (R), Lysine (K);
- 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
- 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

A conservative substitution is a substitution in which the substituting amino acid (naturally occurring or modified) is structurally related to the amino acid being substituted, i.e., has about the same size and electronic properties as the amino acid being substituted. Thus, the substituting amino acid would have the same or a similar functional group in the side chain as the original amino acid. A "conservative substitution" also refers to utilizing a substituting amino acid which is identical to the amino acid being substituted except that a functional group in the side chain is protected with a suitable protecting group.

Suitable protecting groups are described in Green and Wuts, "Protecting Groups in Organic Synthesis", John Wiley and Sons, Chapters 5 and 7, 1991, the teachings of which are incorporated herein by reference. Preferred protecting groups are those which facilitate transport of the peptide through membranes, for example, by reducing the hydrophilicity and increasing the lipophilicity of the peptide, and which can be cleaved, either by hydrolysis or enzymatically (Ditter et al., 1968. J. Pharm. Sci. 57:783; Ditter et al., 1968. J. Pharm. Sci. 57:828; Ditter et al., 1969. J. Pharm. Sci. 58:557; King et al., 1987. Biochemistry 26:2294; Lindberg et al., 1989. Drug



Metabolism and Disposition 17:311; Tunek et al., 1988. Biochem. Pharm. 37:3867; Anderson et al., 1985 Arch. Biochem. Biophys. 239:538; and Singhal et al., 1987. FASEB J. 1:220). Suitable hydroxyl protecting groups include ester, carbonate and carbamate protecting groups. Suitable amine protecting groups include acyl groups and alkoxy or aryloxy carbonyl groups, as described above for N-terminal protecting groups. Suitable carboxylic acid protecting groups include aliphatic, benzyl and aryl esters, as described below for C-terminal protecting groups. In one embodiment, the carboxylic acid group in the side chain of one or more glutamic acid or aspartic acid residues in a peptide of the present invention is protected, preferably as a methyl, ethyl, benzyl or substituted benzyl ester, more preferably as a benzyl ester.

Provided below are groups of naturally occurring and modified amino acids in which each amino acid in a group has similar electronic and steric properties. Thus, a conservative substitution can be made by substituting an amino acid with another amino acid from the same group. Such substitutions may be engineered through selection of the appropriate nucleotides in constructing the gene of interest for introduction into animals receiving gene therapy. It is to be understood that these groups are non-limiting, i.e. that there are additional modified amino acids which could be included in each group.

Group I includes leucine, isoleucine, valine, methionine and modified amino acids having the following side chains: ethyl, n-propyl n-butyl. Preferably, Group I includes leucine, isoleucine, valine and methionine.

Group II includes glycine, alanine, valine and a modified amino acid having an ethyl side chain. Preferably, Group II includes glycine and alanine.

Group III includes phenylalanine, phenylglycine, tyrosine, tryptophan, cyclohexylmethyl glycine, and modified amino residues having substituted benzyl or phenyl side chains. Preferred substituents include one or more of the following: halogen, methyl, ethyl, nitro,  $\text{—NH}_2$ , methoxy, ethoxy and  $\text{—CN}$ . Preferably, Group III includes phenylalanine, tyrosine and tryptophan.

Group IV includes glutamic acid, aspartic acid, a substituted or unsubstituted aliphatic, aromatic or benzylic ester of glutamic or aspartic acid (e.g., methyl, ethyl, n-propyl iso-propyl, cyclohexyl, benzyl or substituted benzyl), glutamine, asparagine,  $\text{—CO—NH—}$  alkylated glutamine or asparagines (e.g., methyl, ethyl, n-propyl and iso-propyl) and modified amino acids having the side chain  $\text{—(CH}_2\text{)}_3\text{—COOH}$ , an ester thereof (substituted or unsubstituted

aliphatic, aromatic or benzylic ester), an amide thereof and a substituted or unsubstituted N-alkylated amide thereof. Preferably, Group IV includes glutamic acid, aspartic acid, methyl aspartate, ethyl aspartate, benzyl aspartate and methyl glutamate, ethyl glutamate and benzyl glutamate, glutamine and asparagine.

Group V includes histidine, lysine, ornithine, arginine, N-nitroarginine,  $\beta$ -cycloarginine,  $\gamma$ -hydroxyarginine, N-amidinocitruline and 2-amino-4-guanidinobutanoic acid, homologs of lysine, homologs of arginine and homologs of ornithine. Preferably, Group V includes histidine, lysine, arginine and ornithine. A homolog of an amino acid includes from 1 to about 3 additional or subtracted methylene units in the side chain.

Group VI includes serine, threonine, cysteine and modified amino acids having C1-C5 straight or branched alkyl side chains substituted with —OH or —SH, for example, —CH<sub>2</sub>CH<sub>2</sub>OH, —CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>OH or —CH<sub>2</sub>CH<sub>2</sub>OHCH<sub>3</sub>. Preferably, Group VI includes serine, cysteine or threonine.

In another aspect, suitable substitutions for amino acid residues include “severe” substitutions. A “severe substitution” is a substitution in which the substituting amino acid (naturally occurring or modified) has significantly different size and/or electronic properties compared with the amino acid being substituted. Thus, the side chain of the substituting amino acid can be significantly larger (or smaller) than the side chain of the amino acid being substituted and/or can have functional groups with significantly different electronic properties than the amino acid being substituted. Examples of severe substitutions of this type include the substitution of phenylalanine or cyclohexylmethyl glycine for alanine, isoleucine for glycine, a D amino acid for the corresponding L amino acid, or —NH—CH[(—CH<sub>2</sub>)<sub>5</sub>—COOH]—CO— for aspartic acid. Alternatively, a functional group may be added to the side chain, deleted from the side chain or exchanged with another functional group. Examples of severe substitutions of this type include adding of valine, leucine or isoleucine, exchanging the carboxylic acid in the side chain of aspartic acid or glutamic acid with an amine, or deleting the amine group in the side chain of lysine or ornithine. In yet another alternative, the side chain of the substituting amino acid can have significantly different steric and electronic properties than the functional group of the amino acid being substituted. Examples of such modifications include tryptophan for glycine, lysine for aspartic acid and —

(CH<sub>2</sub>)<sub>4</sub>COOH for the side chain of serine. These examples are not meant to be limiting.

In another embodiment, for example in the synthesis of a peptide 26 amino acids in length, the individual amino acids may be substituted according in the following manner:

- AA<sub>1</sub> is serine, glycine, alanine, cysteine or threonine;
- AA<sub>2</sub> is alanine, threonine, glycine, cysteine or serine;
- AA<sub>3</sub> is valine, arginine, leucine, isoleucine, methionine, ornithine, lysine, N-nitroarginine, β-cycloarginine, γ-hydroxyarginine, N-amidinocitruline or 2-amino-4-guanidinobutanoic acid;
- AA<sub>4</sub> is proline, leucine, valine, isoleucine or methionine;
- AA<sub>5</sub> is tryptophan, alanine, phenylalanine, tyrosine or glycine;
- AA<sub>6</sub> is serine, glycine, alanine, cysteine or threonine;
- AA<sub>7</sub> is proline, leucine, valine, isoleucine or methionine;
- AA<sub>8</sub> is alanine, threonine, glycine, cysteine or serine;
- AA<sub>9</sub> is alanine, threonine, glycine, cysteine or serine;
- AA<sub>10</sub> is leucine, isoleucine, methionine or valine;
- AA<sub>11</sub> is serine, glycine, alanine, cysteine or threonine;
- AA<sub>12</sub> is leucine, isoleucine, methionine or valine;
- AA<sub>13</sub> is leucine, isoleucine, methionine or valine;
- AA<sub>14</sub> is glutamine, glutamic acid, aspartic acid, asparagine, or a substituted or unsubstituted aliphatic or aryl ester of glutamic acid or aspartic acid;
- AA<sub>15</sub> is arginine, N-nitroarginine, β-cycloarginine, γ-hydroxy-arginine, N-amidinocitruline or 2-amino-4-guanidino-butanoic acid
- AA<sub>16</sub> is proline, leucine, valine, isoleucine or methionine;
- AA<sub>17</sub> is serine, glycine, alanine, cysteine or threonine;
- AA<sub>18</sub> is glutamic acid, aspartic acid, asparagine, glutamine or a substituted or unsubstituted aliphatic or aryl ester of glutamic acid or aspartic acid;
- AA<sub>19</sub> is aspartic acid, asparagine, glutamic acid, glutamine, leucine, valine, isoleucine, methionine or a substituted or unsubstituted aliphatic or aryl ester of glutamic acid or aspartic acid;
- AA<sub>20</sub> is valine, arginine, leucine, isoleucine, methionine, ornithine, lysine, N-nitroarginine, β-cycloarginine, γ-hydroxyarginine, N-amidinocitruline or 2-amino-4-guanidinobutanoic acid;

AA<sub>21</sub> is alanine, threonine, glycine, cysteine or serine;

AA<sub>22</sub> is alanine, threonine, glycine, cysteine or serine;

AA<sub>23</sub> is histidine, serine, threonine, cysteine, lysine or ornithine;

AA<sub>24</sub> is threonine, aspartic acid, serine, glutamic acid or a substituted or unsubstituted  
5 aliphatic or aryl ester of glutamic acid or aspartic acid;

AA<sub>25</sub> is asparagine, aspartic acid, glutamic acid, glutamine, leucine, valine, isoleucine, methionine or a substituted or unsubstituted aliphatic or aryl ester of glutamic acid or aspartic acid; and

AA<sub>26</sub> is cysteine, histidine, serine, threonine, lysine or ornithine.

10 It is to be understood that these amino acid substitutions may be made for longer or shorter peptides than the 26 mer in the preceding example above, and for proteins.

In one embodiment of the present invention, codons for the first several N-terminal amino acids of the transposase are modified such that the third base of each  
15 codon is changed to an A or a T without changing the corresponding amino acid. It is preferable that between approximately 1 and 20, more preferably 3 and 15, and most preferably between 4 and 12 of the first N-terminal codons of the gene of interest are modified such that the third base of each codon is changed to an A or a T without changing the corresponding amino acid. In one embodiment, the first ten N-terminal  
20 codons of the gene of interest are modified in this manner.

When several desired proteins, protein fragments or peptides are encoded in the gene of interest to be incorporated into the genome, one of skill in the art will appreciate that the proteins, protein fragments or peptides may be separated by a spacer molecule such as, for example, a peptide, consisting of one or more amino  
25 acids. Generally, the spacer will have no specific biological activity other than to join the desired proteins, protein fragments or peptides together, or to preserve some minimum distance or other spatial relationship between them. However, the constituent amino acids of the spacer may be selected to influence some property of the molecule such as the folding, net charge, or hydrophobicity. The spacer may also  
30 be contained within a nucleotide sequence with a purification handle or be flanked by cleavage sites, such as proteolytic cleavage sites.

Such polypeptide spacers may have from about 5 to about 40 amino acid residues. The spacers in a polypeptide are independently chosen, but are preferably all the same. The spacers should allow for flexibility of movement in space and are

therefore typically rich in small amino acids, for example, glycine, serine, proline or alanine. Preferably, peptide spacers contain at least 60%, more preferably at least 80% glycine or alanine. In addition, peptide spacers generally have little or no biological and antigenic activity. Preferred spacers are (Gly-Pro-Gly-Gly)<sub>x</sub> (SEQ ID NO:26) and (Gly<sub>4</sub>-Ser)<sub>y</sub>, wherein x is an integer from about 3 to about 9 and y is an integer from about 1 to about 8. Specific examples of suitable spacers include

(Gly-Pro-Gly-Gly)<sub>3</sub>

SEQ ID NO:27 Gly Pro Gly Gly Gly Pro Gly Gly Gly Pro Gly Gly

(Gly<sub>4</sub>-Ser)<sub>3</sub>

10 SEQ ID NO:28 Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser  
or (Gly<sub>4</sub>-Ser)<sub>4</sub>

SEQ ID NO:29 Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser  
Gly Gly Gly Gly Ser.

Nucleotide sequences encoding for the production of residues which may be useful in purification of the expressed recombinant protein in animals receiving gene therapy may also be built into the vector. Such sequences are known in the art and include the glutathione binding domain from glutathione S-transferase, polylysine, hexa-histidine or other cationic amino acids, thioredoxin, hemagglutinin antigen and maltose binding protein.

20 Additionally, nucleotide sequences may be inserted into the gene of interest to be incorporated so that the protein or peptide can also include from one to about six amino acids that create signals for proteolytic cleavage. In this manner, if a gene is designed to make one or more peptides or proteins of interest in the transgenic animal, specific nucleotide sequences encoding for amino acids recognized by enzymes may be incorporated into the gene to facilitate cleavage of the large protein or peptide sequence into desired peptides or proteins or both. For example, nucleotides encoding a proteolytic cleavage site can be introduced into the gene of interest so that a signal sequence can be cleaved from a protein or peptide encoded by the gene of interest. Nucleotide sequences encoding other amino acid sequences which display pH sensitivity or chemical sensitivity may also be added to the vector to facilitate separation of the signal sequence from the peptide or protein of interest.

Proteolytic cleavage sites include cleavage sites recognized by exopeptidases such as carboxypeptidase A, carboxypeptidase B, aminopeptidase I, and dipeptidylaminopeptidase; endopeptidases such as trypsin, V8-protease, enterokinase,

factor Xa, collagenase, endoproteinase, subtilisin, and thrombin; and proteases such as  
Protease 3C IgA protease (Igase) Rhinovirus 3C(preScission)protease . Chemical  
cleavage sites are also included in the definition of cleavage site as used herein.  
Chemical cleavage sites include, but are not limited to, site cleaved by cyanogen  
5 bromide, hydroxylamine, formic acid, and acetic acid.

In one embodiment of the present invention, a TAG sequence is linked to the  
gene of interest. The TAG sequence serves three purposes: 1) it allows free rotation  
of the peptide or protein to be isolated so there is no interference from the native  
protein or signal sequence, i.e. vitellogenin, 2) it provides a “purification handle” to  
10 isolate the protein using column purification, and 3) it includes a cleavage site to  
remove the desired protein from the signal and purification sequences. Accordingly,  
as used herein, a TAG sequence includes a spacer sequence, a purification handle and  
a cleavage site. The spacer sequences in the TAG proteins contain one or more  
repeats shown in SEQ ID NO:30. A preferred spacer sequence comprises the  
15 sequence provided in SEQ ID NO:31. One example of a purification handle is the  
gp41 hairpin loop from HIV I. Exemplary gp41 polynucleotide and polypeptide  
sequences are provided in SEQ ID NO:32 and SEQ ID NO:33, respectively.  
However, it should be understood that any antigenic region may be used as a  
purification handle, including any antigenic region of gp41. Preferred purification  
20 handles are those that elicit highly specific antibodies. Additionally, the cleavage site  
can be any protein cleavage site known to one of ordinary skill in the art and includes  
an enterokinase cleavage site comprising the Asp Asp Asp Asp Lys sequence (SEQ  
ID NO:34) and a furin cleavage site. Constructs containing a TAG sequence are  
shown in Figures 2 and 3. In one embodiment of the present invention, the TAG  
25 sequence comprises a polynucleotide sequence of SEQ ID NO:35.

### Gene Therapy

Administration of the transposon based vectors of the present invention to  
achieve gene therapy in animals may be used to treat numerous genetic and non-  
30 genetic disorders.

DNA constructs of the present invention can be used to transform any animal  
cell, including but not limited to: cells producing hormones, cytokines, growth  
factors, or any other biologically active substance; cells of the immune system; cells  
of the nervous system; muscle (striatal, cardiac, smooth) cells; vascular system cells;

endothelial cells; skin cells; mammary cells; and lung cells, including bronchial and alveolar cells. Transformation of any endocrine cell by a transposon-based DNA construct is contemplated as a part of a present invention. DNA constructs of the present invention can be used to modulate, including both stimulation and inhibition, production of any substance, including but not limited to a hormone, a cytokine, or a growth factor, by an animal cell. Modulation of a regulated signal within a cell or a tissue, such as production of a second messenger, is also contemplated as a part of the present invention. In one aspect of the present invention, cells of the immune system may be the target for incorporation of a desired gene or genes encoding for production of antibodies. Accordingly, the thymus, bone marrow, beta lymphocytes (or B cells), gastrointestinal associated lymphatic tissue (GALT), Peyer's patches, bursa Fabricius, lymph nodes, spleen, and tonsil, and any other lymphatic tissue, may all be targets for administration of the compositions of the present invention. Use of the DNA constructs of the present invention is contemplated for treatment of any animal disease or condition that results from underproduction (such as diabetes) or overproduction (such as hyperthyroidism) of a hormone or other endogenous biologically active substance. Use of DNA constructs of the present invention to integrate nucleotide sequences encoding RNA molecules, such as anti-sense RNA or short interfering RNA, is also contemplated as a part of the present invention.

20

#### Genetic disorders

Genetic disorders are well known to one of ordinary skill in the art and may include, but are not limited to, general classes of mutations, Mendelian disorders, disorders with multifactorial inheritance, cytogenetic disorders, and single gene disorders with nonclassic inheritance.

Mendelian disorders include autosomal dominant disorders autosomal recessive disorders and X-linked disorders. Such disorders may include defective enzymes, defects in receptor and transport systems, alterations in the structure, function or quality of non-enzyme proteins, and genetically determined adverse reactions to drugs. Some of these conditions are related to familial hypercholesterolemia, lysosomal storage diseases, glycogen storage diseases and neurofibromatosis. Provision of gene therapy using the method of the present invention may address, for example, supplementation of an animal with a protein that the animal needs in view of its inadequate or faulty production of the protein.

### Other disorders

The present invention also provides gene therapy for animals that may not possess a demonstrable genetic deficiency. However, such animals may require  
5 supplementation of specific proteins that may be produced in inadequate amounts or in a defective form that renders them biologically inactive or marginally active. Alternatively, animals may produce too much of a protein that causes a disease or condition that renders the animal sick. Such animals may require gene therapy to reduce the transcription of a gene that makes the protein. Such animals may require  
10 gene therapy to produce proteins or peptides to blunt or block the activity of the overabundant protein.

### Diseases and Conditions

Numerous diseases and conditions may be treated with the gene therapy  
15 method of the present invention, including, but not limited to, diseases and conditions of the following systems: cardiovascular system (atherosclerosis, hypercholesterolemia, disorders of LDL, HDL and apolipoprotein synthesis and metabolism, hypertension); reproductive system (reproductive health and dysfunction, fertility, infertility, menopause, menarche, puberty, superovulation, timing of  
20 ovulation, inducement of ovulation, inducement of sterilization (especially of companion animals), mastitis, cancers of the reproductive system); endocrine and neuroendocrine systems (hypopituitary disorders, hypothalamic disorders, hypogonadism, precocious puberty, dwarfism, infertility, lactation, diabetes, thyroid disease, adrenal cortical or adrenal medullary disease, appetite, feeding, drinking,  
25 temperature regulation); metabolic system (digestive disorders, inborn errors of metabolism, disorders of intermediate metabolism, fat metabolism, Crohn's disease; phenylketonuria, chronic wasting disease, phosphofructokinase deficiency, pyruvic kinase deficiency; nervous system (Parkinson's disease, Alzheimer's disease, Huntington's disease, encephalopathy, bovine spongiform encephalopathy, conditions  
30 related to neurotransmitter transporter systems such as catecholamine transporters and reuptake mechanisms (serotonin, norepinephrine, dopamine) such as depression, psychosis, neurosis, addiction, alcoholism, motivation, bulimia, hyperphagia); immune system (feline immunodeficiency virus, simian immunodeficiency virus, immunodeficiency disorders including severe immunodeficiency disorders and severe



combined immunodeficiency disorders, leukemia, autoimmune disorders, allergies, lupus, multiple sclerosis, scleroderma, disorders involving various immunoglobulins, interleukins, cytokines and lymphokines); hematologic and related disorders (sickle cell anemia, clotting disorders, von Willebrand's Disease); musculoskeletal system  
5 (arthritis, rheumatoid arthritis, osteoarthritis, muscular dystrophy); cancer (ovarian, prostate, breast, colon, brain, lung, kidney, skin); respiratory system (lung cancer, laryngeal cancer, cystic fibrosis); obesity; aging; cosmetic treatment of skin and hair; any form of cancer (skin (melanoma, basal, squamous), bladder, colon, stomach, esophageal, liver, pancreatic, testicular, prostate, ovarian, cervical, uterine, breast,  
10 lung, laryngeal, thyroid, adrenal, renal, penile, head, neck, brain (neural, glial); disorders involving receptors, particularly membrane bound receptors; and, infectious diseases (parasitic disease, bacterial infectious disease, viral disease, pneumovirus, Eastern equine encephalitis, West Nile virus, malaria, lyme disease, ehrlichiosis, retroviral infections, rabies, and diseases borne by invertebrates such as ticks, fleas,  
15 flies and mosquitoes.

The transposon-based vectors of the present invention can be used for the treatment of various genetic disorders. For example, one or more LTR-vector complexes can be administered to an animal for the treatment of a single gene disorder including, but not limited to, animal equivalents of Huntington's disease,  
20 alpha-1-antitrypsin deficiency Alzheimer's disease, various forms of breast cancer, cystic fibrosis, galactosemia, congenital hypothyroidism, maple syrup urine disease, neurofibromatosis 1, phenylketonuria, sickle cell disease, and Smith-Lemli-Opitz (SLO/RSH) Syndrome any metabolic errors, autoimmune diseases, shipping fever in cattle, mastitis, bacterial or viral diseases, alteration of skin pigment in animals,  
25 production of animals with enhanced growth characteristics and nutrient utilization. In these embodiments, the transposon-based vector contains a non-mutated, or non-disease causing form of the gene known to cause such disorder. The transposon-based vectors of the present invention can also be used to treat multiple gene disorders. The transposon-based vectors of the present invention can be used as DNA  
30 vaccines and are useful in organ-specific disease treatments and localized disease treatments.

Preferably, the transposase contained within the transposase-based vector is operably linked to an inducible promoter such as a tissue specific promoter such that the non-mutated gene of interest is inserted into a specific tissue wherein the mutated

gene is expressed *in vivo*. Additionally, the DNA constructs of the present invention can be used to provide cells or tissues with “beacons”, such as receptor molecules, for binding of therapeutic agents in order to provide tissue and cell specificity for the therapeutic agents. Several promoters and exogenous genes can be combined in one  
5 vector to produce progressive, controlled, treatments, from a single vector delivery.

In avians, for example, one or more LTR-vector complexes are administered to an avian for the treatment of a viral or bacterial infection/disease including, but not limited to, Colibacillosis (Coliform infections), Mycoplasmosis (CRD, Air sac, Sinusitis), Fowl Cholera, Necrotic Enteritis, Ulcerative Enteritis (Quail disease),  
10 Pullorum Disease, Fowl Typhoid, Botulism, Infectious Coryza, Erysipelas, Avian Pox, Newcastle Disease, Infectious Bronchitis, Quail Bronchitis, Lymphoid Leukosis, Marek's Disease (Visceral Leukosis), Infectious Bursal Disease (Gumboro), Avian Encephalomyelitis (AE, Avian Influenza (AI), Avian Leukosis Virus (LLAg, LLAb, ALV-J), Reticuloendotheliosis Virus (REV), Avian Pneumovirus (APV), Chicken  
15 Anemia Virus (CAV), Infectious Bronchitis Virus (IBV), Infectious Bursal Disease Virus – Gumboro Disease (IBD, IBD-XR), Mycoplasma (MG, MS, MG/MS, MM), Newcastle Disease Virus (NDV, NDV-T), Ornithobacterium rhinotracheale (ORT), Pasteurella multocida (PM, PM-T), Reovirus (REO), and Salmonella enteritidis (SE).

In swine, for example, one or more transposon-based vectors are administered  
20 for the treatment of a viral or bacterial infection/disease including, but not limited to, Pseudorabies Virus – Aujeszky's Disease (PRV-V, PRV-S, PRV gl (gE)), Porcine Reproductive and Respiratory Syndrome (PRRS 2XR), Classical Swine Fever Virus (CSFV Ab, CSFV Ag), Swine Influenza (SIV H1N1), Mycoplasma hyopneumoniae (M. hyo.), and Swine Salmonella.

In ruminants, for example, one or more transposon-based vectors are administered for the treatment of a viral or bacterial infection/disease including, but not limited to, Bovine Leukemia Virus (BLV), Infectious Bovine Rhinotracheitis (IBR, IBR gB, IBR gE), Brucella abortus (B. abortus), Mycobacterium paratuberculosis – Johne's Disease (M. pt.), Neospora caninum, and Bovine Viral  
25 Diarrhea Virus (BVDV).  
30

In horses, for example, one or more transposon-based vectors are administered for the treatment of a viral or bacterial infection/disease including, but not limited to, Equine Infectious Anemia (EIA).

### Methods of Administering Transposon-Based Vectors

The compositions of the present invention, comprising a vector, a transfecting reagent and an acceptable carrier may be delivered to a desired location in an animal receiving gene therapy through administration via a selected route. Accordingly, the compositions may be administered in a variety of ways including, but not limited to the following: through a vascular system, a duct system, within the lumen of an organ, into an organ, into a body cavity, into the cerebrospinal fluid, topically, through the gastrointestinal system, through the reproductive system, through the urinary system, intraperitoneally, and through the respiratory system.

The compositions may be delivered through the vascular system to be distributed to the cells supplied by that vessel. For example, the compositions may be placed in the artery supplying the ovary or to the fallopian tube to supply those tissues. In this manner, follicles could be transfected to create a germline transgenic animal. Alternatively, supplying the compositions through the artery leading to the fallopian tube would preferably transfect the epithelial cells. Such transfected epithelial cells could manufacture a desired protein or peptide for deposition in the egg white. Administration of the compositions through the portal vein would target uptake and transformation of hepatic cells. Administration through the urethra and into the bladder would target the transitional epithelium of the bladder. Administration through the vagina and cervix would target the lining of the uterus. Administration through the internal mammary artery would transfect secretory cells of the lactating mammary gland to perform a desired function, such as to synthesize and secrete a desired protein or peptide into the milk. Administration through the internal mammary artery would also target breast cancer cells.

In one embodiment of the present invention, a transposon-based vector comprising a gene encoding proinsulin is administered to diabetic animals receiving gene therapy for incorporation into liver cells in order to treat or cure diabetes. The specific incorporation of the proinsulin gene into the liver is accomplished by placing the transposase of the transposon-based vector under control of liver-specific promoter, such as the glucose-6-phosphatase promoter (G6P). This approach is useful for treatment of both type I and type II diabetes. The G6P promoter has been shown to be glucose responsive (Arguad, D., et al. 1996, *Diabetes* 45: 1563-1571), and thus, glucose-regulated insulin production is achieved using DNA constructs of the present

invention. Integrating a proinsulin gene into liver cells circumvents the problem of destruction of pancreatic islet cells in the course of type 1 diabetes.

In another embodiment, shortly after diagnosis of type I diabetes, the cells of the immune system destroying  $\beta$ -cells of the pancreas are selectively removed using the DNA constructs of the present invention, thus allowing normal  $\beta$ -cells to repopulate the pancreas.

For treatment of type II diabetes, the DNA constructs of the present invention are specifically incorporated into the pancreas by placing the transposase of the transposon-based vector under the control of a pancreas-specific promoter, such as an insulin promoter. In this embodiment, the vector is delivered to a diabetic animal via injection into an artery supplying the pancreas. For delivery, the vector is complexed with a transfection agent. The artery distributes the complex throughout the pancreas, where individual cells receive the vector DNA. Following uptake into the target cell, the insulin promoter is recognized by transcriptional machinery of the cell, the transposase encoded by the vector is expressed, and stable integration of the proinsulin gene occurs. It is expected that a small percentage of the DNA construct would be transported to other tissues, and that these tissues would be transfected. However, these tissues would not be stably transfected due to failure of these other cells to activate the insulin promoter. The DNA would likely be lost when the cell dies or degraded over time.

In addition to the transposon-based vectors described above, the present invention also includes methods of administering the transposon-based vectors to an animal, methods of producing a transgenic animal wherein a gene of interest is incorporated into the germline of the animal and methods of producing a transgenic animal wherein a gene of interest is incorporated into cells other than the germline cells (somatic cells) of the animal. For example, the transposon-based vectors of the present invention are administered to a reproductive organ of an animal via any method known to those of skill in the art. Preferred reproductive organs include a testis, an ovary, an oviduct, a mammary gland, and a fallopian tube.

In some embodiments, a transposon-based vector is directly administered to the reproductive organ. Direct administration encompasses injection into the organ, and in one embodiment, a transposon-based vector is injected into the lumen of the oviduct, and more preferably, the lumen of the magnum or the infundibulum of the

oviduct. The transposon-based vectors may additionally or alternatively be placed in an artery supplying the reproductive organ. Administering the vectors to the artery supplying the ovary results in transfection of follicles and oocytes in the ovary to create a germline transgenic animal. Alternatively, supplying the vectors through an artery leading to the oviduct would preferably transfect the tubular gland and epithelial cells. Such transfected cells manufacture a desired protein or peptide for deposition in the egg white. In one embodiment, a transposon-based vector is administered into the lumen of the magnum or the infundibulum of the oviduct and to an artery supplying the oviduct. Indirect administration to the oviduct epithelium may occur through the cloaca. Direct administration into the mammary gland may be achieved through introduction into the duct system of the mammary gland or an artery supplying the mammary gland.

The transposon-based vectors may be administered in a single administration, multiple administrations, continuously, or intermittently. The transposon-based vectors may be administered by injection, via a catheter, an osmotic mini-pump or any other method. In some embodiments, the transposon-based vector is administered to an animal in multiple administrations, each administration containing the vector and a different transfecting reagent.

The transposon-based vectors may be administered to the animal at any desirable time for gene therapy during the lifetime of the animal.

In one embodiment, between approximately 1  $\mu$ g and 5 mg, 1  $\mu$ g and 3 mg, 1  $\mu$ g and 1 mg, of transposon-based vector DNA is administered to the animal. Intraoviduct administration of the transposon-based vectors of the present invention resulted in incorporation of the gene of interest into the cells of the oviduct as evidenced by a PCR positive signal in the oviduct tissue, demonstrating that the present invention is effective in providing genetic therapy to the animal. In other embodiments, the transposon-based vector is administered to an artery that supplies the oviduct. These methods of administration may also be combined with any methods for facilitating transfection, including without limitation, electroporation, gene guns, injection of naked DNA, and use of dimethyl sulfoxide (DMSO).

According to the present invention, the transposon-based vector is administered in conjunction with an acceptable carrier and/or transfection reagent. Acceptable carriers include, but are not limited to, water, saline, Hanks Balanced Salt

Solution (HBSS), Tris-EDTA (TE) and lyotropic liquid crystals. Transfection reagents commonly known to one of ordinary skill in the art that may be employed include, but are not limited to, the following: cationic lipid transfection reagents, cationic lipid mixtures, polyamine reagents, liposomes and combinations thereof; SUPERFECT®, Cytofectene, BioPORTER®, GenePORTER®, NeuroPORTER®, and perfectin from Gene Therapy Systems; lipofectamine, cellfectin, DMRIE-C oligofectamine, TROJENE® and PLUS reagent from InVitrogen; Xtreme gene, fugene, DOSPER and DOTAP from Roche; Lipotaxi and Genejammer from Strategene; and Escort from SIGMA. In one embodiment, the transfection reagent is SUPERFECT®. The ratio of DNA to transfection reagent may vary based upon the method of administration. In one embodiment, the transposon-based vector is administered to the oviduct and the ratio of DNA to transfection reagent can be from 1:1.5 to 1:15, preferably 1:2 to 1:5, all expressed as wt/vol. Transfection may also be accomplished using other means known to one of ordinary skill in the art, including without limitation electroporation, gene guns, injection of naked DNA, and use of dimethyl sulfoxide (DMSO).

Depending upon the cell or tissue type targeted for transfection, the form of the transposon-based vector may be important. Plasmids harvested from bacteria are generally closed circular supercoiled molecules, and this is the preferred state of a vector for gene delivery because of the ease of preparation. In some instances, transposase expression and insertion may be more efficient in a relaxed, closed circular configuration or in a linear configuration. In still other instances, a purified transposase protein may be co-injected with a transposon-based vector containing the gene of interest for more immediate insertion. This could be accomplished by using a transfection reagent complexed with both the purified transposase protein and the transposon-based vector.

#### Testing for and Breeding Animals Carrying the Transgene

Following administration of a transposon-based vector to an animal receiving gene therapy, DNA is extracted from the animal to confirm integration of the gene of interest. Advantages provided by the present invention include the high rates of integration, or incorporation, and transcription of the gene of interest when administered to a bird via an intraoviduct or intraovarian route (including intraarterial administrations to arteries leading to the oviduct or ovary). Example 6 below

describes isolation of a proinsulin/ENT TAG protein from a transgenic hen following ammonium sulfate precipitation and ion exchange chromatography. Figure 5 demonstrates successful administration of a transposon-based vector to a hen, successful integration of the gene of interest, successful production of a protein encoded by the gene of interest, and successful deposition of the protein in egg white produced by the transgenic hen.

Actual frequencies of integration may be estimated both by comparative strength of the PCR signal, and by histological evaluation of the tissues by quantitative PCR. Another method for estimating the rate of transgene insertion is the so-called primed *in situ* hybridization technique (PRINS). This method determines not only which cells carry a transgene of interest, but also into which chromosome the gene has inserted, and even what portion of the chromosome. Briefly, labeled primers are annealed to chromosome spreads (affixed to glass slides) through one round of PCR, and the slides are then developed through normal *in situ* hybridization procedures. This technique combines the best features of *in situ* PCR and fluorescence *in situ* hybridization (FISH) to provide distinct chromosome location and copy number of the gene in question.

Breeding experiments are also conducted to determine if germline transmission of the transgene has occurred. In a general bird breeding experiment performed according to the present invention, each male bird was exposed to 2-3 different adult female birds for 3-4 days each. This procedure was continued with different females for a total period of 6-12 weeks. Eggs are collected daily for up to 14 days after the last exposure to the transgenic male, and each egg is incubated in a standard incubator. The resulting embryos are examined for transgene presence at day 3 or 4 using PCR. It is to be understood that the above procedure can be modified to suit animals other than birds and that selective breeding techniques may be performed to amplify gene copy numbers and protein output.

#### Production of Desired Proteins or Peptides in Egg White

In one embodiment, the transposon-based vectors of the present invention may be administered to a bird receiving gene therapy for production of desired proteins or peptides in the egg white. These transposon-based vectors preferably contain one or more of an ovalbumin promoter, an ovomucoid promoter, an ovalbumin signal sequence and an ovomucoid signal sequence. Oviduct-specific ovalbumin promoters

are described in B. O'Malley et al., 1987. EMBO J., vol. 6, pp. 2305-12; A. Qiu et al., 1994. Proc. Nat. Acad. Sci. (USA), vol. 91, pp. 4451-4455; D. Monroe et al., 2000. Biochim. Biophys. Acta, 1517 (1):27-32; H. Park et al., 2000. Biochem., 39:8537-8545; and T. Muramatsu et al., 1996. Poult. Avian Biol. Rev., 6:107-123. Examples  
5 of transposon-based vectors designed for production of a desired protein in an egg white are shown in Figures 2 and 3.

#### Production of Desired Proteins or Peptides in Egg Yolk

The present invention is particularly advantageous for production of  
10 recombinant peptides and proteins of low solubility in the egg yolk. Such proteins include, but are not limited to, membrane-associated or membrane-bound proteins, lipophilic compounds; attachment factors, receptors, and components of second messenger transduction machinery. Low solubility peptides and proteins are particularly challenging to produce using conventional recombinant protein  
15 production techniques (cell and tissue cultures) because they aggregate in water-based, hydrophilic environments. Such aggregation necessitates denaturation and re-folding of the recombinantly-produced proteins, which may deleteriously affect their structure and function. Moreover, even highly soluble recombinant peptides and proteins may precipitate and require denaturation and renaturation when produced in  
20 sufficiently high amounts in recombinant protein production systems. The present invention provides an advantageous resolution of the problem of protein and peptide solubility during production of large amounts of recombinant proteins.

In one embodiment of the present invention wherein germline transfection is obtained via intraovarian administration of the transposon-based vector, deposition of  
25 a desired protein into the egg yolk is accomplished in offspring by attaching a sequence encoding a protein capable of binding to the yolk vitellogenin receptor to a gene of interest that encodes a desired protein. This transposon-based vector can be used for the receptor-mediated uptake of the desired protein by the oocytes. In a preferred embodiment, the sequence ensuring the binding to the vitellogenin receptor  
30 is a targeting sequence of a vitellogenin protein. The invention encompasses various vitellogenin proteins and their targeting sequences. In a preferred embodiment, a chicken vitellogenin protein targeting sequence is used, however, due to the high degree of conservation among vitellogenin protein sequences and known cross-species reactivity of vitellogenin targeting sequences with their egg-yolk receptors,



other vitellogenin targeting sequences can be substituted. One example of a construct for use in the transposon-based vectors of the present invention and for deposition of an insulin protein in an egg yolk is a transposon-based vector containing a vitellogenin promoter, a vitellogenin targeting sequence, a TAG sequence, a pro-insulin sequence and a synthetic polyA sequence. The present invention includes, but is not limited to, vitellogenin targeting sequences residing in the N-terminal domain of vitellogenin, particularly in lipovitellin I. In one embodiment, the vitellogenin targeting sequence contains the polynucleotide sequence of SEQ ID NO:22. In a preferred embodiment, the transposon-based vector contains a transposase gene operably-linked to a constitutive promoter and a gene of interest operably-linked to a liver-specific promoter and a vitellogenin targeting sequence.

The following examples will serve to further illustrate the present invention without, at the same time, however, constituting any limitation thereof. On the contrary, it is to be clearly understood that resort may be had to various embodiments, modifications and equivalents thereof which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the invention.

#### EXAMPLE 1

##### *20 IntraOviduct Administration of Transposon-Based Vectors*

Quail or chicken were selected for administration of the transposon-based vectors of the present invention. Feathers were removed from the area where surgery was performed and the area was cleansed and sterilized by rinsing it with ethanol (alcohol) and 0.5% chlorhexidine. Using the scalpel, a dorsolateral incision was made through the skin over the ovary approximately 2 cm in length. Using blunt scissors, a second incision was made through the muscle between the last two ribs to expose the oviduct beneath. A small animal retractor was used to spread the last two ribs, exposing the oviduct beneath. The oviduct was further exposed using retractors to pull the intestines to one side.

30 A delivery solution containing a transposon-based vector and SUPERFECT® was prepared fresh immediately before surgery. Specific ratios of vector and SUPERFECT® that were used in each experiment are provided in the Examples below. The delivery solution was warmed to room temperature prior to injection into

the bird. Approximately 250-500 µl of the delivery solution was injected into the lumen of the magnum of the oviduct using a 1 cc syringe with a 27 gauge needle attached. The wound was closed and antibiotic cream liberally applied to the area surrounding the wound.

5

## EXAMPLE 2

### *Preparation of Transposon-Based Vector pTnMod*

A vector was designed for inserting a desired coding sequence into the genome of eukaryotic cells, given below as SEQ ID NO:3. The vector of SEQ ID  
10 NO:3, termed pTnMod, was constructed and its sequence verified.

This vector employed a cytomegalovirus (CMV) promoter. A modified Kozak sequence (ACCATG) (SEQ ID NO:1) was added to the promoter. The nucleotide in the wobble position in nucleotide triplet codons encoding the first 10 amino acids of transposase was changed to an adenine (A) or thymine (T), which did not alter the  
15 amino acid encoded by this codon. Two stop codons were added and a synthetic polyA was used to provide a strong termination sequence. This vector uses a promoter designed to be active soon after entering the cell (without any induction) to increase the likelihood of stable integration. The additional stop codons and synthetic polyA insures proper termination without read through to potential genes  
20 downstream.

The first step in constructing this vector was to modify the transposase to have the desired changes. Modifications to the transposase were accomplished with the primers High Efficiency forward primer (Hef) Altered transposase (ATS)-Hef 5' ATCTCGAGACCATGTGTGAACTTGATATTTTACATGATTCTCTTTACC 3'  
25 (SEQ ID NO:36) and Altered transposase- High efficiency reverse primer (Her) 5' GATTGATCATTATCATAATTTCCCCAAAGCGTAACC 3' (SEQ ID NO:37, a reverse complement primer). In the 5' forward primer ATS-Hef, the sequence CTCGAG (SEQ ID NO:38) is the recognition site for the restriction enzyme Xho I, which permits directional cloning of the amplified gene. The sequence ACCATG  
30 (SEQ ID NO:1) contains the Kozak sequence and start codon for the transposase and the underlined bases represent changes in the wobble position to an A or T of codons for the first 10 amino acids (without changing the amino acid coded by the codon). Primer ATS-Her (SEQ ID NO:37) contains an additional stop codon TAA in addition

to native stop codon TGA and adds a Bcl I restriction site, TGATCA (SEQ ID NO:39), to allow directional cloning. These primers were used in a PCR reaction with pTnLac (p defines plasmid, tn defines transposon, and lac defines the beta fragment of the lactose gene, which contains a multiple cloning site) as the template  
5 for the transposase and a FailSafe™ PCR System (which includes enzyme, buffers, dNTPs, MgCl<sub>2</sub> and PCR Enhancer; Epicentre Technologies, Madison, WI). Amplified PCR product was electrophoresed on a 1% agarose gel, stained with ethidium bromide, and visualized on an ultraviolet transilluminator. A band corresponding to the expected size was excised from the gel and purified from the  
10 agarose using a Zymo Clean Gel Recovery Kit (Zymo Research, Orange, CA). Purified DNA was digested with restriction enzymes Xho I (5') and Bcl I (3') (New England Biolabs, Beverly, MA) according to the manufacturer's protocol. Digested DNA was purified from restriction enzymes using a Zymo DNA Clean and Concentrator kit (Zymo Research).

15 Plasmid gWhiz (Gene Therapy Systems, San Diego, CA) was digested with restriction enzymes Sal I and BamH I (New England Biolabs), which are compatible with Xho I and Bcl I, but destroy the restriction sites. Digested gWhiz was separated on an agarose gel, the desired band excised and purified as described above. Cutting the vector in this manner facilitated directional cloning of the modified transposase  
20 (mATS) between the CMV promoter and synthetic polyA.

To insert the mATS between the CMV promoter and synthetic polyA in gWhiz, a Stratagene T4 Ligase Kit (Stratagene, Inc. La Jolla, CA) was used and the ligation set up according to the manufacturer's protocol. Ligated product was transformed into *E. coli* Top10 competent cells (Invitrogen Life Technologies,  
25 Carlsbad, CA) using chemical transformation according to Invitrogen's protocol. Transformed bacteria were incubated in 1 ml of SOC (GIBCO BRL, CAT# 15544-042) medium for 1 hour at 37° C before being spread to LB (Luria-Bertani media (broth or agar)) plates supplemented with 100 µg/ml ampicillin (LB/amp plates). These plates were incubated overnight at 37° C and resulting colonies picked to  
30 LB/amp broth for overnight growth at 37° C. Plasmid DNA was isolated using a modified alkaline lysis protocol (Sambrook et al., 1989), electrophoresed on a 1% agarose gel, and visualized on a U.V. transilluminator after ethidium bromide staining. Colonies producing a plasmid of the expected size (approximately 6.4 kbp)

were cultured in at least 250 ml of LB/amp broth and plasmid DNA harvested using a Qiagen Maxi-Prep Kit (column purification) according to the manufacturer's protocol (Qiagen, Inc., Chatsworth, CA). Column purified DNA was used as template for sequencing to verify the changes made in the transposase were the desired changes  
5 and no further changes or mutations occurred due to PCR amplification. For sequencing, Perkin-Elmer's Big Dye Sequencing Kit was used. All samples were sent to the Gene Probes and Expression Laboratory (LSU School of Veterinary Medicine) for sequencing on a Perkin-Elmer Model 377 Automated Sequencer.

Once a clone was identified that contained the desired mATS in the correct  
10 orientation, primers CMVf-NgoM IV (5' TTGCCGGCATCAGATTGGCTAT (SEQ ID NO:40); underlined bases denote a NgoM IV recognition site) and Syn-polyA-BstE II (5' AGAGGTCACCGGGTC AATTCTTCAGCACCTGGTA (SEQ ID NO:41); underlined bases denote a BstE II recognition site) were used to PCR amplify the entire CMV promoter, mATS, and synthetic polyA for cloning upstream of the  
15 transposon in pTnLac. The PCR was conducted with FailSafe<sup>TM</sup> as described above, purified using the Zymo Clean and Concentrator kit, the ends digested with NgoM IV and BstE II (New England Biolabs), purified with the Zymo kit again and cloned upstream of the transposon in pTnLac as described below.

Plasmid pTnLac was digested with NgoM IV and BstE II to remove the ptac  
20 promoter and transposase and the fragments separated on an agarose gel. The band corresponding to the vector and transposon was excised, purified from the agarose, and dephosphorylated with calf intestinal alkaline phosphatase (New England Biolabs) to prevent self-annealing. The enzyme was removed from the vector using a Zymo DNA Clean and Concentrator-5. The purified vector and CMVp/mATS/polyA  
25 were ligated together using a Stratagene T4 Ligase Kit and transformed into *E. coli* as described above.

Colonies resulting from this transformation were screened (mini-preps) as describe above and clones that were the correct size were verified by DNA sequence analysis as described above. The vector was given the name pTnMod (SEQ ID NO:3)  
30 and includes the following components:

Base pairs 1-130 are a remainder of F1(-) on from pBluescriptII sk(-) (Stratagene), corresponding to base pairs 1-130 of pBluescriptII sk(-).

Base pairs 131 - 132 are a residue from ligation of restriction enzyme sites used in constructing the vector.

Base pairs 133 -1777 are the CMV promoter/enhancer taken from vector pGWiz (Gene Therapy Systems), corresponding to bp 229-1873 of pGWiz. The CMV promoter was modified by the addition of an ACC sequence upstream of ATG.

Base pairs 1778-1779 are a residue from ligation of restriction enzyme sites  
5 used in constructing the vector.

Base pairs 1780 - 2987 are the coding sequence for the transposase, modified from Tn10 (GenBank accession J01829) by optimizing codons for stability of the transposase mRNA and for the expression of protein. More specifically, in each of the codons for the first ten amino acids of the transposase, G or C was changed to A or T  
10 when such a substitution would not alter the amino acid that was encoded.

Base pairs 2988-2993 are two engineered stop codons.

Base pair 2994 is a residue from ligation of restriction enzyme sites used in constructing the vector.

Base pairs 2995 - 3410 are a synthetic polyA sequence taken from the pGWiz  
15 vector (Gene Therapy Systems), corresponding to bp 1922-2337 of 10 pGWiz.

Base pairs 3415 - 3718 are non-coding DNA that is residual from vector pNK2859.

Base pairs 3719 - 3761 are non-coding  $\lambda$  DNA that is residual from pNK2859.

Base pairs 3762 - 3831 are the 70 bp of the left insertion sequence recognized  
20 by the transposon Tn10.

Base pairs 3832-3837 are a residue from ligation of restriction enzyme sites used in constructing the vector.

Base pairs 3838 - 4527 are the multiple cloning site from pBluescriptII sk(20), corresponding to bp 924-235 of pBluescriptII sk(-). This multiple cloning site may be  
25 used to insert any coding sequence of interest into the vector.

Base pairs 4528-4532 are a residue from ligation of restriction enzyme sites used in constructing the vector.

Base pairs 4533 - 4602 are the 70 bp of the right insertion sequence recognized by the transposon Tn10.

Base pairs 4603 - 4644 are non-coding  $\lambda$  DNA that is residual from pNK2859.  
30

Base pairs 4645 - 5488 are non-coding DNA that is residual from pNK2859.

Base pairs 5489 - 7689 are from the pBluescriptII sk(-) base vector - (Stratagene, Inc.), corresponding to bp 761-2961 of pBluescriptII sk(-).

Completing pTnMod is a pBlueScript backbone that contains a colE 1 origin of replication and an antibiotic resistance marker (ampicillin).

It should be noted that all non-coding DNA sequences described above can be replaced with any other non-coding DNA sequence(s). Missing nucleotide sequences  
5 in the above construct represent restriction site remnants.

All plasmid DNA was isolated by standard procedures. Briefly, *Escherichia coli* containing the plasmid was grown in 500 mL aliquots of LB broth (supplemented with an appropriate antibiotic) at 37°C overnight with shaking. Plasmid DNA was recovered from the bacteria using a Qiagen Maxi-Prep kit (Qiagen, Inc., Chatsworth,  
10 CA) according to the manufacturer's protocol. Plasmid DNA was resuspended in 500 µL of PCR-grade water and stored at -20°C until used.

### EXAMPLE 3

#### *Transposon-Based Vector pTnMCS*

15 Another transposon-based vector was designed for inserting a desired coding sequence into the genome of eukaryotic cells. This vector was termed pTnMCS and its constituents are provided below. The sequence of the pTnMCS vector is provided in SEQ ID NO:2. The pTnMCS vector contains an avian optimized polyA sequence operably-linked to the transposase gene. The avian optimized polyA sequence  
20 contains approximately 40 nucleotides that precede the A nucleotide string.

Bp 1 – 130 Remainder of F1 (-) ori of pBluescriptII sk(-) (Stratagene) bp1-130

Bp 133 – 1777 CMV promoter/enhancer taken from vector pGWIZ (Gene Therapy Systems) bp 229-1873

Bp 1783 – 2991 Transposase, from Tn10 (GenBank accession #J01829) bp 108-1316

25 Bp 2992 – 3344 Non coding DNA from vector pNK2859

Bp 3345 – 3387 Lambda DNA from pNK2859

Bp 3388 – 3457 70 bp of IS10 left from Tn10

Bp 3464 – 3670 Multiple cloning site from pBluescriptII sk(-), thru the XmaI site bp 924-718

30 Bp 3671 - 3715 Multiple cloning site from pBluescriptII sk(-), from the XmaI site thru the XhoI site. These base pairs are usually lost when cloning into pTnMCS bp 717-673

Bp 3716 – 4153 Multiple cloning site from pBluescriptII sk(-), from the XhoI site bp 672-235

Bp 4159 - 4228 70 bp of IS10 right from Tn10

Bp 4229 - 4270 Lambda DNA from pNK2859

Bp 4271 – 5114 Non-coding DNA from pNK2859

Bp 5115 - 7315 pBluescript sk (-) base vector (Stratagene, Inc.) bp 761-2961.

5

#### EXAMPLE 4

##### *Preparation of Transposon-Based Vector pTnMod(Oval/ENT TAG/ProIns/PA) – Chicken*

10 A vector was designed to insert a human proinsulin coding sequence under the control of a chicken ovalbumin promoter, and a ovalbumin gene including an ovalbumin signal sequence, into the genome of a bird given below as SEQ ID NO:42.

Base pairs 1 - 130 are a remainder of F1(-) ori of pBluescriptII sk(-) (Stratagene) corresponding to base pairs 1-130 of pBluescriptII sk(-).

15 Base pairs 133 – 1777 are a CMV promoter/enhancer taken from vector pGWiz (Gene Therapy Systems) corresponding to base pairs 229-1873 of pGWiz.

Base pairs 1780 – 2987 are a transposase, modified from Tn10 (GenBank accession number J01829).

Base pairs 2988-2993 are two engineered stop codons.

20 Base pairs 2995 – 3410 are a synthetic polyA from pGWiz (Gene Therapy Systems) corresponding to base pairs 1922- 2337 of pGWiz.

Base pairs 3415 – 3718 are non coding DNA that is residual from vector pNK2859.

Base pairs 3719 – 3761 are  $\lambda$  DNA that is residual from pNK2859.

25 Base pairs 3762 – 3831 are the 70 base pairs of the left insertion sequence (IS10) recognized by the transposon Tn10.

Base pairs 3838 – 4044 are a multiple cloning site from pBlueScriptII sk(-) corresponding to base pairs 924-718 of pBluescriptII sk(-).

30 Base pairs 4050 - 4951 are a chicken ovalbumin promoter (including SDRE) that corresponds to base pairs 431-1332 of the chicken ovalbumin promoter in GenBank Accession Number J00895 M24999.

Base pairs 4958 - 6115 are a chicken ovalbumin signal sequence and ovalbumin gene that correspond to base pairs 66-1223 of GenBank Accession Number V00383.1. (The STOP codon being omitted).

Base pairs 6122 - 6271 are a TAG sequence containing a gp41 hairpin loop from HIV I, an enterokinase cleavage site and a spacer (synthetic).

Base pairs 6272 – 6531 are a proinsulin gene.

Base pairs 6539 – 6891 are a synthetic polyadenylation sequence from pGWiz (Gene Therapy Systems) corresponding to base pairs 1920 - 2272 of pGWiz.

Base pairs 6897 - 7329 are a multiple cloning site from pBlueScriptII sk(-) corresponding to base pairs 667-235 of pBluescriptII sk(-).

Base pairs 7335- 7404 are the 70 base pairs of the right insertion sequence (IS10) recognized by the transposon Tn10.

Base pairs 7405 - 7446 are  $\lambda$  DNA that is residual from pNK2859.

Base pairs 7447 – 8311 are non coding DNA that is residual from pNK2859.

Base pairs 8312 - 10512 are pBlueScript sk(-) base vector (Stratagene, Inc.) corresponding to base pairs 761-2961 of pBluescriptII sk(-).

It should be noted that all non-coding DNA sequences described above can be replaced with any other non-coding DNA sequence(s). Missing nucleotide sequences in the above construct represent restriction site remnants.

#### EXAMPLE 5

##### *Transposon-Based Vector pTnMOD (CMV-CHOVg-ent-ProInsulin-synPA)*

A vector was designed to insert a proinsulin coding sequence under the control of a quail ovalbumin promoter, and a ovalbumin gene including an ovalbumin signal sequence, into the genome of a bird given below as SEQ ID NO:43.

Bp 1 – 4045 from vector pTnMod, bp 1 - 4045

Bp 4051 – 5695 CMV promoter/enhancer taken from vector pGWIZ (Gene therapy systems), bp 230-1864

Bp 5702 –6855 Chicken ovalbumin gene taken from GenBank accession # V00383, bp 66-1219

Bp 6862 - 7011 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site

Bp 7012 – 7272 Human Proinsulin taken from GenBank accession # NM000207, bp 117-377

Bp 7273 – 7317 Spacer DNA, derived as an artifact from the cloning vectors pTOPO



Blunt II (Invitrogen) and pGWIZ (Gene Therapy Systems)

Bp 7318 - 7670 Synthetic polyA from the cloning vector pGWIZ (Gene Therapy Systems), bp 1920-2271

Bp 7672 –11271 from cloning vector pTnMCS, bp 3716-7315

5

#### EXAMPLE 6

##### *Transfection of Japanese Quail using a Transposon-based Vector containing a Proinsulin Gene via Oviduct Injections*

Two experiments were conducted in Japanese quail using transposon-based  
10 vectors containing either Oval promoter/Oval gene/GP41 Enterokinase  
TAG/Proinsulin/Poly A (SEQ ID NO:42) or CMV promoter/Oval gene/GP41  
Enterokinase TAG/Proinsulin/Poly A (SEQ ID NO:43).

In the first experiment, the Oval promoter/Oval gene/GP41 Enterokinase  
TAG/Proinsulin/Poly A containing construct was injected into the lumen of the  
15 oviduct of sexually mature quail; three hens received 5 µg at a 1:3 SUPERFECT®  
ratio and three received 10 µg at a 1:3 SUPERFECT® ratio. As of the writing of the  
present application, at least one bird that received above-mentioned construct was  
producing human proinsulin in egg white (other birds remain to be tested). This  
experiment indicates that 1) the DNA has been stable for at least 3 months; 2) protein  
20 levels are comparable to those observed with a constitutive promoter such as the  
CMV promoter; and 3) sexually mature birds can be injected and results obtained  
without the need for cell culture. It is estimated that each quail egg contains  
approximately 1.4 µg/ml of the proinsulin protein. It is also estimated that each  
transgenic chicken egg contains 50-75 mg of protein encoded by the gene of interest.

25 In the second experiment, the transposon-based vector containing CMV  
promoter/Oval gene/GP41 Enterokinase TAG/Proinsulin/Poly A was injected into the  
lumen of the oviduct of sexually immature Japanese quail. A total of 9 birds were  
injected. Of the 8 survivors, 3 produced human proinsulin in the white of their eggs  
for over 6 weeks. An ELISA assay described in detail below was developed to detect  
30 GP41 in the fusion peptide (Oval gene/GP41 Enterokinase TAG/Proinsulin) since the  
GP41 peptide sequence is unique and not found as part of normal egg white protein.  
In all ELISA assays, the same birds produced positive results and all controls worked  
as expected.

ELISA Procedure: Individual egg white samples were diluted in sodium carbonate buffer, pH 9.6, and added to individual wells of 96 well microtiter ELISA plates at a total volume of 0.1 ml. These plates were then allowed to coat overnight at 4°C. Prior to ELISA development, the plates were allowed warm to room  
5 temperature. Upon decanting the coating solutions and blotting away any excess, non-specific binding of antibodies was blocked by adding a solution of phosphate buffered saline (PBS), 1% (w/v) BSA, and 0.05% (v/v) Tween 20 and allowing it to incubate with shaking for a minimum of 45 minutes. This blocking solution was subsequently decanted and replaced with a solution of the primary antibody (Goat  
10 Anti-GP41 TAG) diluted in fresh PBS/BSA/Tween 20. After a two hour period of incubation with the primary antibody, each plate was washed with a solution of PBS and 0.05% Tween 20 in an automated plate washer to remove unbound antibody. Next, the secondary antibody, Rabbit anti-Goat Alkaline Phosphatase-conjugated, was diluted in PBS/BSA/Tween 20 and allowed to incubate 1 hour. The plates were then  
15 subjected to a second wash with PBS/Tween 20. Antigen was detected using a solution of *p*-Nitrophenyl Phosphate in Diethanolamine Substrate Buffer for Alkaline Phosphatase and measuring the absorbance at 30 minutes and 1 hour.

Additionally, a proinsulin fusion protein produced using a construct described above was isolated from egg white using ammonium sulfate precipitation and ion  
20 exchange chromatography. A pooled fraction of the isolated fusion protein was run on an SDS-PAGE gel shown in Figure 5, lanes 4 and 6. Lanes 1 and 10 of the gel contain molecular weight standards, lanes 2 and 8 contain non-transgenic chicken egg white, whereas lanes 3, 5, 7 and 9 are blank.

25

## EXAMPLE 7

### *Isolation of Human Proinsulin Using Anti-TAG Column Chromatography*

A HiTrap NHS-activated 1 mL column (Amersham) was charged with a 30 amino acid peptide that contained the gp-41 epitope containing gp-41's native disulfide bond that stabilizes the formation of the gp-41 hairpin loop. The 30 amino  
30 acid gp41 peptide is provided as SEQ ID NO:32. Approximately 10 mg of the peptide was dissolved in coupling buffer (0.2 M NaHCO<sub>3</sub>, 0.5 M NaCl, pH 8.3 and the ligand was circulated on the column for 2 hours at room temperature at 0.5 mL/minute. Excess active groups were then deactivated using 6 column volumes of

0.5 M ethanolamine, 0.5 M NaCl, pH 8.3 and the column was washed alternately with 6 column volumes of acetate buffer (0.1 M acetate, 0.5 M NaCl, pH 4.0) and ethanolamine (above). The column was neutralized using 1 X PBS. The column was then washed with buffers to be used in affinity purification: 75 mM Tris, pH 8.0 and  
5 elution buffer, 100 mM glycine-HCl, 0.5 M NaCl, pH 2.7. Finally, the column was equilibrated in 75 mM Tris buffer, pH 8.0.

Antibodies to gp-41 were raised in goats by inoculation with the gp-41 peptide described above. More specifically, goats were inoculated, given a booster injection of the gp-41 peptide and blood samples were obtained by venipuncture. Serum was  
10 harvested by centrifugation. Approximately 30 mL of goat serum was filtered to 0.45 uM and passed over a TAG column at a rate of 0.5 mL/min. The column was washed with 75 mM Tris, pH 8.0 until absorbance at 280 nm reached a baseline. Three column volumes (3 mL) of elution buffer (100 mM glycine, 0.5 M NaCl, pH 2.7) was applied, followed by 75 mM Tris buffer, pH 8.0, all at a rate of 0.5 mL/min. One ml  
15 fractions were collected. Fractions were collected into 200 uL 1 M Tris, pH 9.0 to neutralize acidic fractions as rapidly as possible. A large peak eluted from the column, coincident with the application the elution buffer. Fractions were pooled. Analysis by SDS-PAGE showed a high molecular weight species that separated into two fragments under reducing condition, in keeping with the heavy and light chain  
20 structure of IgG.

Pooled antibody fractions were used to charge two 1 mL HiTrap NHS-activated columns, attached in series. Coupling was carried out in the same manner as that used for charging the TAG column.

## 25 Isolation of Ovalbumin-TAG-Proinsulin from Egg White

Egg white from quail and chickens treated by intra-oviduct injection of the CMV-ovalbumin-TAG-proinsulin construct were pooled. Viscosity was lowered by subjecting the allantoic fluid to successively finer pore sizes using negative pressure filtration, finishing with a 0.22  $\mu$ M pore size. Through the process, egg white was  
30 diluted approximately 1:16. The clarified sample was loaded on the Anti-TAG column and eluted in the same manner as described for the purification of the anti-TAG antibodies. A peak of absorbance at 280 nm, coincident with the application of

the elution buffer, indicated that protein had been specifically eluted from the Anti-TAG column. Fractions containing the eluted peak were pooled for analysis.

The pooled fractions from the Anti-TAG affinity column were characterized by SDS-PAGE and Western blot analysis. SDS-PAGE of the pooled fractions  
5 revealed a 60 kDal molecular weight band not present in control egg white fluid, consistent with the predicted molecular weight of the transgenic protein. Although some contaminating bands were observed, the 60 kDal species was greatly enriched compared to the other proteins. An aliquot of the pooled fractions was cleaved overnight at room temperature with the protease, enterokinase. SDS-PAGE analysis  
10 of the cleavage product, revealed a band not present in the uncut material that co-migrated with a commercial human proinsulin positive control. Western blot analysis showed specific binding to the 60 kDal species under non-reducing condition (which preserved the hairpin epitope of gp-41 by retaining the disulfide bond). Western analysis of the low molecular weight species that appeared upon cleavage with an  
15 anti-human proinsulin antibody, conclusively identified the cleaved fragment as human proinsulin.

## EXAMPLE 8

### *Purification Procedures for Insulin*

#### 20 I. ELISA data for egg characterization/identification

An ELISA was employed for the initial screening of eggs and, thereby, identification of hens producing positive eggs. With further modifications this procedure was used for the initial quantification of recombinant protein amounts. These procedures were aided by the successful purification of an initial stock of the  
25 recombinant proinsulin (RPI). This stock of protein is used in the development of a double antibody assay that increases the sensitivity and reduces the background in the assay. Subsequent identification of hens producing positive eggs obviate the need to screen each egg collected. Only periodic checks are needed to determine if production levels are consistent.

#### 30 II. Egg White (EW) or Albumin Preparation

##### A. Clarification – Ovomucin precipitation

Eggs from hens positively identified as producing RPI are pooled for RPI purification. The initial purification step involved diluting the pool 1:1 with 100 mM Tris-HCl, pH 8 for a final concentration of 50 mM Tris-HCl. The pH of this solution

was then adjusted to 6 and ovomucin was allowed to precipitate at 4°C for a minimum of 3hrs (preferably overnight) with constant stirring. The precipitated ovomucin was then pelleted and removed by centrifugation at 2400 x g. After collection of the RPI containing supernatant, the pH of this solution was readjusted to 8.

## 5           B.       Filtration

To prepare the egg white for loading onto the column and, thereby, minimize the potential for clogging the columns during loading, the egg white solution was filtered to at least 0.45  $\mu$ m.

Initially, the ovomucin precipitated egg white solution was subjected to  
10 successive filtration steps with the pore size of the filtration membrane decreasing at each step. This procedure involved time and dilution of the egg white solution to reach 0.45  $\mu$ m filtration.

Amersham's hollow-fiber ultrafiltration apparatus was used to produce a  
column-ready solution filtered down to < 0.2  $\mu$ m with an undiluted starting solution.  
15 This approach minimized the time and the solution dilution needed to prepare the egg white solution for column loading.

## III. Purification

### A.       Affinity Chromatography –

Using antibody with specificity to a synthetic peptide modeled after the  
20 enterokinase recognition site, initial purification schemes involved developing a one-step column purification procedure for the RPI.

Goats immunized with the synthetic Ent peptide were employed to produce anti-Ent Tag antiserum which was used in the egg screening ELISAs followed by antibody purification. The purified goat Anti-Ent Tag antibodies were covalently  
25 bound to the matrix of HiTrap NHS-activated HP columns (Amersham) and subsequently used to specifically bind and purify the RPI.

An initial attempt was made to direct the first purification step against the ovalbumin portion of the recombinant protein using an antibody specific for the ovalbumin portion. The present purification scheme employed a combination of  
30 classical techniques such as ammonium sulfate precipitation, ion exchange, and gel filtration chromatography.

After the initial ovomucin precipitation, the egg white solution was subjected to protein precipitation using a 40% ammonium sulfate fractionation. The

precipitated protein was subsequently collected via centrifugation and resuspended in 50 mM Tris-HCl, pH 8. The resuspended protein solution was dialyzed to remove residual  $(\text{NH}_4)_2\text{SO}_4$  or subjected to gel filtration to remove the  $(\text{NH}_4)_2\text{SO}_4$  and partially isolate the RPI from the remaining egg white protein. The RPI was further  
5 isolated via anion exchange chromatography using a 0 to 0.5M NaCl gradient in 50 mM Tris-HCl, pH 8. Two possible elution profiles were observed. One at approximately 25% of the 0.5 M NaCl gradient without  $(\text{NH}_4)_2\text{SO}_4$  precipitation. The second was observed at less than 16% gradient (approximately 7%) following 40%  $(\text{NH}_4)_2\text{SO}_4$  precipitation and a longer gradient. Fractions containing RPI were  
10 identified by SDS-PAGE analysis and pooled.

Three gel filtration columns, differing by column size and fractionation range, were employed in RPI purification and/or desalting: Superdex 75 10/300 GL, Hiload 26/60 Superdex 75, and Hiload 26/60 Superdex 200. Using these individual columns at different steps in the purification scheme increased the efficiency of the process.  
15 Fractions containing RPI were identified by SDS-PAGE analysis and pooled.

Cleavage of the RPI Enterokinase recognition site was accomplished using purified enterokinase from Sigma. Enterokinase, 0.004 Unit/ $\mu\text{l}$  per reaction, was applied to the pooled and, if necessary, concentrated protein solution. The digestion reaction was incubated at room temperature (up to 30°C in a rolling hybridization  
20 oven) for a minimum of 16 h and in some cases up to 48 hrs of incubation. The digestion efficiency was followed using 16.5% Tris-Tricine SDS-PAGE peptide gels. All gel staining utilized Simply Blue Coomassie Staining Solutions. Free Proinsulin was observed on gels after digestion.

A subsequent gel filtration separation was employed to obtain purified  
25 Proinsulin, and to remove the remaining Ovalbumin portion of the RPI and residual native EW proteins. Select steps in the purification process were analyzed using the 2-dimensional Beckman Coulter ProteomeLab PF2D Protein Fractionation System.

## EXAMPLE 9

### 30 *Optimization of Intra-oviduct and Intra-ovarian Arterial Injections*

Overall transfection rates of oviduct cells in a flock of chicken or quail hens are enhanced by synchronizing the development of the oviduct and ovary within the flock. When the development of the oviducts and ovaries are uniform across a group

of hens and when the stage of oviduct and ovarian development can be determined or predicted, timing of injections is optimized to transfect the greatest number of cells. Accordingly, oviduct development is synchronized as described below to ensure that a large and uniform proportion of oviduct secretory cells are transfected with the gene of interest.

Hens are treated with estradiol to stimulate oviduct maturation as described in Oka and Schimke (T. Oka and RT Schimke, *J. Cell Biol.*, 41, 816 (1969)), Palmiter, Christensen and Schimke (*J Biol. Chem.* 245(4):833-845, 1970). Specifically, repeated daily injections of 1 mg estradiol benzoate are performed sometime before the onset of sexual maturation, a period ranging from 1 – 14 weeks of age. After a stimulation period sufficient to maximize development of the oviduct, hormone treatment is withdrawn thereby causing regression in oviduct secretory cell size but not cell number. At an optimum time after hormone withdrawal, the lumens of the oviducts of treated hens are injected with the transposon-based vector. Hens are subjected to additional estrogen stimulation after an optimized time during which the transposon-based vector is taken up into oviduct secretory cells. Re-stimulation by estrogen activates transposon expression, causing the integration of the gene of interest into the host genome. Estrogen stimulation is then withdrawn and hens continue normal sexual development. If a developmentally regulated promoter such as the ovalbumin promoter is used, expression of the transposon-based vector initiates in the oviduct at the time of sexual maturation. Intra-ovarian artery injection during this window allows for high and uniform transfection efficiencies of ovarian follicles to produce germ-line transfections and possibly oviduct expression.

Other means are also used to synchronize the development, or regression, of the oviduct and ovary to allow high and uniform transfection efficiencies. Alterations of lighting and/or feed regimens, for example, cause hens to ‘molt’ during which time the oviduct and ovary regress. Molting is used to synchronize hens for transfection, and may be used in conjunction with other hormonal methods to control regression and/or development of the oviduct and ovary.

## EXAMPLE 10

### *Preparation of Transposon-Based Vector pTnMod(Oval/ENT TAG/ProIns/PA) – Quail*

5           A vector is designed for inserting a proinsulin gene under the control of a quail ovalbumin promoter, and a ovalbumin gene including an ovalbumin signal sequence, into the genome of a bird given below as SEQ ID NO:44.

          Base pairs 1 -130 are a remainder of F1(-) ori of pBluescriptII sk(-)  
10 (Stratagene) corresponding to base pairs 1-130 of pBluescriptII sk(-).

          Base pairs 133 – 1777 are a CMV promoter/enhancer taken from vector pGWiz (Gene Therapy Systems) corresponding to base pairs 229-1873 of pGWiz.

          Base pairs 1780 – 2987 are a transposase, modified from Tn10 (GenBank accession number J01829).

15           Base pairs 2988-2993 are an engineered stop codon.

          Base pairs 2995 – 3410 are a synthetic polyA from pGWiz (Gene Therapy Systems) corresponding to base pairs 1922- 2337 of pGWiz.

          Base pairs 3415 – 3718 are non coding DNA that is residual from vector pNK2859.

20           Base pairs 3719 – 3761 are  $\lambda$  DNA that is residual from pNK2859.

          Base pairs 3762 – 3831 are the 70 base pairs of the left insertion sequence (IS10) recognized by the transposon Tn10.

          Base pairs 3838 – 4044 are a multiple cloning site from pBlueScriptII sk(-) corresponding to base pairs 924-718 of pBluescriptII sk(-).

25           Base pairs 4050 - 4938 are the Japanese quail ovalbumin promoter (including SDRE, steroid-dependent response element). The Japanese quail ovalbumin promoter was isolated by its high degree of homology to the chicken ovalbumin promoter (GenBank accession number J00895 M24999, base pairs 431-1332). Some deletions were noted in the quail sequence, as compared to the chicken sequence.

30           Base pairs 4945 - 6092 are a quail ovalbumin signal sequence and ovalbumin gene that corresponds to base pairs 54 – 1201 of GenBank accession number X53964.1. (The STOP codon being omitted).

          Base pairs 6093 - 6246 are a TAG sequence containing a gp41 hairpin loop from HIV I an enterokinase cleavage site and a spacer (synthetic).



Base pairs 6247 – 6507 are a proinsulin gene.

Base pairs 6514 – 6866 are a synthetic polyadenylation sequence from pGWiz (Gene Therapy Systems) corresponding to base pairs 1920 - 2272 of pGWiz.

Base pairs 6867 - 7303 are a multiple cloning site from pBlueScriptII sk(-)  
5 corresponding to base pairs 667-235 of pBluescriptII sk(-).

Base pairs 7304- 7379 are the 70 base pairs of the right insertion sequence (IS10) recognized by the transposon Tn10.

Base pairs 7380 - 7421 are  $\lambda$  DNA that is residual from pNK2859.

Base pairs 7422 – 8286 are non coding DNA that is residual from pNK2859.

10 Base pairs 8287 - 10487 are pBlueScript sk(-) base vector (Stratagene, Inc.) corresponding to base pairs 761-2961 of pBluescriptII sk(-).

It should be noted that all non-coding DNA sequences described above can be replaced with any other non-coding DNA sequence(s). Missing nucleotide sequences in the above construct represent restriction site remnants.

15

#### EXAMPLE 11

##### *Preparation of Transposon-Based Vector pTnMod(Oval/ENT TAG/p146/PA) – Chicken*

A vector was designed for inserting a p146 gene under the control of a chicken  
20 ovalbumin promoter, and a ovalbumin gene including an ovalbumin signal sequence, into the genome of a bird. The vector sequence is provided below as SEQ ID NO:45.

Base pairs 1 - 130 are a remainder of F1(-) ori of pBluescriptII sk(-) (Stratagene) corresponding to base pairs 1-130 of pBluescriptII sk(-).

Base pairs 133 – 1777 are a CMV promoter/enhancer taken from vector  
25 pGWiz (Gene Therapy Systems) corresponding to base pairs 229-1873 of pGWiz.

Base pairs 1780 – 2987 are a transposase, modified from Tn10 (GenBank accession number J01829).

Base pairs 2988-2993 are an engineered stop codon.

Base pairs 2995 – 3410 are a synthetic polyA from pGWiz (Gene Therapy  
30 Systems) corresponding to base pairs 1922- 2337 of pGWiz.

Base pairs 3415 – 3718 are non coding DNA that is residual from vector pNK2859.

Base pairs 3719 – 3761 are  $\lambda$  DNA that is residual from pNK2859.

Base pairs 3762 – 3831 are the 70 base pairs of the left insertion sequence (IS10) recognized by the transposon Tn10.

Base pairs 3838 – 4044 are a multiple cloning site from pBlueScriptII sk(-) corresponding to base pairs 924-718 of pBluescriptII sk(-).

5        Base pairs 4050 - 4951 are a chicken ovalbumin promoter (including SDRE, steroid-dependent response element) that corresponds to base pairs 431-1332 of the chicken ovalbumin promoter in GenBank Accession Number J00895 M24999.

Base pairs 4958 - 6115 are a chicken ovalbumin signal sequence and Ovalbumin gene that correspond to base pairs 66-1223 of GenBank Accession  
10    Number V00383.1 (The STOP codon being omitted).

Base pairs 6122 - 6271 are a TAG sequence containing a gp41 hairpin loop from HIV I, an enterokinase cleavage site and a spacer (synthetic).

Base pairs 6272 – 6316 are a p146 sequence (synthetic) with 2 added stop codons.

15        Base pairs 6324 – 6676 are a synthetic polyadenylation sequence from pGWiz (Gene Therapy Systems) corresponding to base pairs 1920 - 2272 of pGWiz.

Base pairs 6682 - 7114 are a multiple cloning site from pBlueScriptII sk(-) corresponding to base pairs 667-235 of pBluescriptII sk(-).

Base pairs 7120- 7189 are the 70 base pairs of the right insertion sequence  
20    (IS10) recognized by the transposon Tn10.

Base pairs 7190 - 7231 are  $\lambda$  DNA that is residual from pNK2859.

Base pairs 7232 – 8096 are non coding DNA that is residual from pNK2859.

Base pairs 8097 - 10297 are pBlueScript sk(-) base vector (Stratagene, Inc.) corresponding to base pairs 761-2961 of pBluescriptII sk(-).

25

It should be noted that all non-coding DNA sequences described above can be replaced with any other non-coding DNA sequence(s). Missing nucleotide sequences in the above construct represent restriction site remnants.

30

## EXAMPLE 12

### *Preparation of Transposon-Based Vector pTnMod(Oval/ENT TAG/p146/PA) – Quail*

A vector was designed for inserting a p146 gene under the control of a quail ovalbumin promoter, and a ovalbumin gene including an ovalbumin signal sequence, into the genome of a bird. The vector sequence is given below as SEQ ID NO:46.

Base pairs 1 - 130 are a remainder of F1(-) ori of pBluescriptII sk(-) (Stratagene) corresponding to base pairs 1-130 of pBluescriptII sk(-).

Base pairs 133 – 1777 are a CMV promoter/enhancer taken from vector pGWiz (Gene Therapy Systems) corresponding to base pairs 229-1873 of pGWiz.

5       Base pairs 1780 – 2987 are a transposase, modified from Tn10 (GenBank accession number J01829).

Base pairs 2988-2993 are an engineered stop codon.

Base pairs 2995 – 3410 are a synthetic polyA from pGWiz (Gene Therapy Systems) corresponding to base pairs 1922-2337 of pGWiz.

10       Base pairs 3415 – 3718 are non coding DNA that is residual from vector pNK2859.

Base pairs 3719 – 3761 are  $\lambda$  DNA that is residual from pNK2859.

Base pairs 3762 – 3831 are the 70 base pairs of the left insertion sequence (IS10) recognized by the transposon Tn10.

15       Base pairs 3838 – 4044 are a multiple cloning site from pBlueScriptII sk(-) corresponding to base pairs 924-718 of pBluescriptII sk(-).

Base pairs 4050 - 4938 are the Japanese quail ovalbumin promoter (including SDRE, steroid-dependent response element). The Japanese quail ovalbumin promoter was isolated by its high degree of homology to the chicken ovalbumin promoter (GenBank accession number J00895 M24999, base pairs 431-1332).

20       Bp 4945 - 6092 are a quail ovalbumin signal sequence and ovalbumin gene that corresponds to base pairs 54 – 1201 of GenBank accession number X53964.1. (The STOP codon being omitted).

Base pairs 6097 - 6246 are a TAG sequence containing a gp41 hairpin loop from HIV I, an enterokinase cleavage site and a spacer (synthetic).

25       Base pairs 6247 – 6291 are a p146 sequence (synthetic) with 2 added stop codons.

Base pairs 6299 – 6651 are a synthetic polyadenylation sequence from pGWiz (Gene Therapy Systems) corresponding to base pairs 1920 - 2272 of pGWiz.

30       Base pairs 6657 - 7089 are a multiple cloning site from pBlueScriptII sk(-) corresponding to base pairs 667-235 of pBluescriptII sk(-).

Base pairs 7095- 7164 are the 70 base pairs of the right insertion sequence (IS10) recognized by the transposon Tn10.

Base pairs 7165 - 7206 are  $\lambda$  DNA that is residual from pNK2859.

Base pairs 7207 – 8071 are non coding DNA that is residual from pNK2859.

Base pairs 8072 - 10272 are pBlueScript sk(-) base vector (Stratagene, Inc.) corresponding to base pairs 761-2961 of pBluescriptII sk(-).

- 5           It should be noted that all non-coding DNA sequences described above can be replaced with any other non-coding DNA sequence(s). Missing nucleotide sequences in the above construct represent restriction site remnants.

### EXAMPLE 13

10   *Additional Transposon-Based Vectors for Administration to an Animal*

The following example provides a description of various transposon-based vectors of the present invention and several constructs that have been made for insertion into the transposon-based vectors of the present invention. These examples are not meant to be limiting in any way. The constructs for insertion into a transposon-based vector are provided in a cloning vector pTnMCS or pTnMod, both described above.

pTnMCS (CMV-CHOVg-ent-ProInsulin-synPA) (SEQ ID NO:47)

Bp 1 – 3670 from vector PTnMCS, bp 1 - 3670

- 20   Bp 3676 – 5320 CMV promoter/enhancer taken from vector pGWIZ (Gene Therapy Systems), bp 230-1864

Bp 5327 – 6480 Chicken ovalbumin gene taken from GenBank accession # V00383, bp 66-1219

- 25   Bp 6487 - 6636 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site

Bp 6637 – 6897 Human Proinsulin taken from GenBank accession # NM000207, bp 117-377

Bp 6898 – 6942 Spacer DNA, derived as an artifact from the cloning vectors pTOPO Blunt II (Invitrogen) and pGWIZ (Gene Therapy Systems)

- 30   Bp 6943 - 7295 Synthetic polyA from the cloning vector pGWIZ (Gene Therapy Systems), bp 1920-2271

Bp 7296 – 10895 from cloning vector pTnMCS, bp 3716-7315

pTnMCS (CMV-prepro-ent-ProInsulin-synPA)

Bp 1 – 3670 from vector pTnMCS, bp 1 - 3670

Bp 3676 – 5320 CMV promoter/enhancer taken from vector pGWIZ (Gene Therapy Systems), bp 230-1864

- 5 Bp 5326 - 5496 Capsite/prepro taken from GenBank accession # X07404, bp 563–733  
Bp 5504 - 5652 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site

Bp 5653 – 5913 Human Proinsulin taken from GenBank accession # NM000207, bp 117-377

- 10 Bp 5914 – 5958 Spacer DNA, derived as an artifact from the cloning vectors pTOPO Blunt II (Invitrogen) and pGWIZ (Gene Therapy Systems)  
Bp 5959-6310 Synthetic polyA from the cloning vector pGWIZ (Gene Therapy Systems), bp 1920-2271  
Bp 6313–9912 from cloning vector pTnMCS, bp 3716-7315

15

pTnMCS(Chicken OVep+OVg'+ENT+proins+syn polyA)

Bp 1–3670 from vector pTnMCS, bp 1 - 3670

Bp 3676–4350 Chicken Ovalbumin enhancer taken from GenBank accession #S82527.1 bp 1-675

- 20 Bp 4357–5692 Chicken Ovalbumin promoter taken from GenBank accession # J00895M24999 bp 1-1336

Bp 5699–6917 Chicken Ovalbumin gene from GenBank Accession # V00383.1 bp 2-1220. (This sequence includes the 5'UTR, containing putative cap site, bp 5699-5762.)

- 25 Bp 6924-7073 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site

Bp 7074-7334 Human proinsulin GenBank Accession # NM000207 bp 117-377

Bp 7335-7379 Spacer DNA, derived as an artifact from the cloning vectors pTOPO Blunt II (Invitrogen) and gWIZ (Gene Therapy Systems)

- 30 Bp 7380-7731 Synthetic polyA from the cloning vector gWIZ (Gene Therapy Systems) bp 1920 - 2271

Bp 7733–11332 from vector pTnMCS, bp 3716 - 7315

pTnMCS(Chicken OVep+prepro+ENT+proins+syn polyA)

Bp 1 – 3670 from cloning vector pTnMCS, bp 1 - 3670

Bp 3676 – 4350 Chicken Ovalbumin enhancer taken from GenBank accession # S82527.1 bp 1-675

5 Bp 4357 – 5692 Chicken Ovalbumin promoter taken from GenBank accession # J00895-M24999 bp 1-1336

Bp 5699–5869 Cecropin cap site and prepro, Genbank accession # X07404 bp 563-733

10 Bp 5876 - 6025 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site

Bp 6026 - 6286 Human proinsulin GenBank Accession # NM000207 bp 117-377

Bp 6287 - 6331 Spacer DNA, derived as an artifact from the cloning vectors pTOPO Blunt II (Invitrogen) and gWIZ (Gene Therapy Systems)

15 Bp 6332 - 6683 Synthetic polyA from the cloning vector gWIZ (Gene Therapy Systems) bp 1920 - 2271

Bp 6685 – 10284 from cloning vector pTnMCS, bp 3716 - 7315

pTnMCS(Quail OVep+OVg'+ENT+proins+syn polyA)

Bp 1 – 3670 from cloning vector pTnMCS, bp 1 - 3670

20 Bp 3676 – 4333 Quail Ovalbumin enhancer: 658 bp sequence, amplified in-house from quail genomic DNA, roughly equivalent to the far-upstream chicken ovalbumin enhancer, GenBank accession # S82527.1, bp 1-675. (There are multiple base pair substitutions and deletions in the quail sequence, relative to chicken, so the number of bases does not correspond exactly.)

25 Bp 4340 – 5705 Quail Ovalbumin promoter: 1366 bp sequence, amplified in-house from quail genomic DNA, roughly corresponding to chicken ovalbumin promoter, GenBank accession # J00895-M24999 bp 1-1336. (There are multiple base pair substitutions and deletions between the quail and chicken sequences, so the number of bases does not correspond exactly.)

30 Bp 5712 – 6910 Quail Ovalbumin gene, EMBL accession # X53964, bp 1-1199. (This sequence includes the 5'UTR, containing putative cap site bp 5712-5764.)

Bp 6917 - 7066 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site

Bp 7067 - 7327 Human proinsulin GenBank Accession # NM000207 bp 117-377

Bp 7328 - 7372 Spacer DNA, derived as an artifact from the cloning vectors pTOPO Blunt II (Invitrogen) and gWIZ (Gene Therapy Systems)

Bp 7373 - 7724 Synthetic polyA from the cloning vector gWIZ (Gene Therapy Systems) bp 1920 - 2271

5 Bp 7726 – 11325 from cloning vector pTnMCS, bp 3716 - 7315

pTnMCS(Quail OVep+prepro+ENT+proins+syn polyA)

Bp 1 – 3670 from cloning vector pTnMCS, bp 1 - 3670

10 Bp 3676 – 4333 Quail Ovalbumin enhancer: 658 bp sequence, amplified from quail genomic DNA, roughly equivalent to the far- upstream chicken ovalbumin enhancer, GenBank accession #S82527.1, bp 1-675. (There are multiple base pair substitutions and deletions in the quail sequence, relative to chicken, so the number of bases does not correspond exactly.)

15 Bp 4340 – 5705 Quail Ovalbumin promoter: 1366 bp sequence, amplified from quail genomic DNA, roughly corresponding to chicken ovalbumin promoter, GenBank accession # J00895-M24999 bp 1-1336. (There are multiple base pair substitutions and deletions between the quail and chicken sequences, so the number of bases does not correspond exactly.)

20 Bp 5712–5882 Cecropin cap site and prepro, Genbank accession # X07404 bp 563-733

Bp 5889 - 6038 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site

Bp 6039 - 6299 Human proinsulin GenBank Accession # NM000207 bp 117-377

25 Bp 6300 - 6344 Spacer DNA, derived as an artifact from the cloning vectors pTOPO Blunt II (Invitrogen) and gWIZ (Gene Therapy Systems)

Bp 6345 - 6696 Synthetic polyA from the cloning vector gWIZ (Gene Therapy Systems) bp 1920 - 2271

Bp 6698 – 10297 from cloning vector pTnMCS, bp 3716 - 7315.

30 pTnMOD (CMV-prepro-ent-proins-synPA)

Bp 1 – 4045 from vector PTnMCS, bp 1 - 4045

Bp 4051 – 5695 CMV promoter/enhancer taken from vector pGWIZ (Gene therapy systems), bp 230-1864

Bp 5701-5871 Capsite/prepro taken from GenBank accession # X07404, bp 563–733

- Bp 5879 - 6027 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site
- Bp 6028–6288 Human Proinsulin taken from GenBank accession # NM000207, bp 117-377
- 5 Bp 6289 – 6333 Spacer DNA, derived as an artifact from the cloning vectors pTOPO Blunt II (Invitrogen) and pGWIZ (Gene Therapy Systems)
- Bp 6334 - 6685 Synthetic polyA from the cloning vector pGWIZ (Gene Therapy Systems), bp 1920-2271
- Bp 6687 –10286 from cloning vector pTnMCS, bp 3716-7315
- 10 pTnMOD(Chicken OVep+OVg'+ENT+proins+syn polyA)
- Bp 1 – 4045 from cloning vector pTnMod, bp 1 - 4045
- Bp 4051 – 4725 Chicken Ovalbumin enhancer taken from GenBank accession # S82527.1 bp 1-675
- 15 Bp 4732 – 6067 Chicken Ovalbumin promoter taken from GenBank accession # J00895-M24999 bp 1-1336
- Bp 6074 – 7292 Chicken Ovalbumin gene from GenBank Accession # V00383.1 bp 2-1220. (This sequence includes the 5'UTR, containing putative cap site bp 6074-6137.)
- 20 Bp 7299 - 7448 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site
- Bp 7449 - 7709 Human proinsulin GenBank Accession # NM000207 bp 117-377
- Bp 7710 - 7754 Spacer DNA, derived as an artifact from the cloning vectors pTOPO Blunt II (Invitrogen) and gWIZ (Gene Therapy Systems)
- 25 Bp 7755 - 8106 Synthetic polyA from the cloning vector gWIZ (Gene Therapy Systems) bp 1920 - 2271
- Bp 8108 – 11707 from cloning vector pTnMod, bp 3716 - 7315
- pTnMOD(Chicken OVep+prepro+ENT+proins+syn polyA)
- 30 Bp 1 – 4045 from cloning vector pTnMCS, bp 1 - 4045
- Bp 4051 – 4725 Chicken Ovalbumin enhancer taken from GenBank accession # S82527.1 bp 1-675
- Bp 4732 – 6067 Chicken Ovalbumin promoter taken from GenBank accession # J00895-M24999 bp 1-1336



- Bp 6074–6244 Cecropin cap site and prepro, Genbank accession # X07404 bp 563-733
- Bp 6251 - 6400 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site
- 5 Bp 6401 - 6661 Human proinsulin GenBank Accession # NM000207 bp 117-377
- Bp 6662 - 6706 Spacer DNA, derived as an artifact from the cloning vectors pTOPO Blunt II (Invitrogen) and gWIZ (Gene Therapy Systems)
- Bp 6707 - 7058 Synthetic polyA from the cloning vector gWIZ (Gene Therapy Systems) bp 1920 - 2271
- 10 Bp 7060 – 10659 from cloning vector pTnMCS, bp 3716 - 7315
- pTnMOD(Quail OVep+OVg'+ENT+proins+syn polyA)
- Bp 1 – 4045 from cloning vector pTnMCS, bp 1 - 4045
- Bp 4051 – 4708 Quail Ovalbumin enhancer: 658 bp sequence, amplified in-house
- 15 from quail genomic DNA, roughly equivalent to the far-upstream chicken ovalbumin enhancer, GenBank accession # S82527.1, bp 1-675. (There are multiple base pair substitutions and deletions in the quail sequence, relative to chicken, so the number of bases does not correspond exactly.)
- Bp 4715 – 6080 Quail Ovalbumin promoter: 1366 bp sequence, amplified in-house
- 20 from quail genomic DNA, roughly corresponding to chicken ovalbumin promoter, GenBank accession # J00895-M24999 bp 1-1336. (There are multiple base pair substitutions and deletions between the quail and chicken sequences, so the number of bases does not correspond exactly.)
- Bp 6087 – 7285 Quail Ovalbumin gene, EMBL accession # X53964, bp 1-1199. (This
- 25 sequence includes the 5'UTR, containing putative cap site bp 6087-6139.)
- Bp 7292 - 7441 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site
- Bp 7442 - 7702 Human proinsulin GenBank Accession # NM000207 bp 117-377
- Bp 7703 - 7747 Spacer DNA, derived as an artifact from the cloning vectors pTOPO
- 30 Blunt II (Invitrogen) and gWIZ (Gene Therapy Systems)
- Bp 7748 - 8099 Synthetic polyA from the cloning vector gWIZ (Gene Therapy Systems) bp 1920 - 2271
- Bp 8101 – 11700 from cloning vector pTnMCS, bp 3716 - 7315

pTnMOD(Quail OVep+prepro+ENT+proins+syn polyA)

Bp 1 – 4045 from cloning vector pTnMCS, bp 1 - 4045

Bp 4051 – 4708 Quail Ovalbumin enhancer: 658 bp sequence, amplified in-house from quail genomic DNA, roughly equivalent to the far-upstream chicken ovalbumin enhancer, GenBank accession #S82527.1, bp 1-675. (There are multiple base pair substitutions and deletions in the quail sequence, relative to chicken, so the number of bases does not correspond exactly.)

Bp 4715 – 6080 Quail Ovalbumin promoter: 1366 bp sequence, amplified in-house from quail genomic DNA, roughly corresponding to chicken ovalbumin promoter, GenBank accession # J00895-M24999 bp 1-1336. (There are multiple base pair substitutions and deletions between the quail and chicken sequences, so the number of bases does not correspond exactly.)

Bp 6087–6257 Cecropin cap site and Prepro, Genbank accession # X07404 bp 563-733

Bp 6264 - 6413 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site

Bp 6414 - 6674 Human proinsulin GenBank Accession # NM000207 bp 117-377

Bp 6675 - 6719 Spacer DNA, derived as an artifact from the cloning vectors pTOPO Blunt II (Invitrogen) and gWIZ (Gene Therapy Systems)

Bp 6720 - 7071 Synthetic polyA from the cloning vector gWIZ (Gene Therapy Systems) bp 1920 - 2271

Bp 7073 – 10672 from cloning vector pTnMCS, bp 3716 - 7315

pTnMOD (CMV-prepro-ent-hGH-CPA)

Bp 1–4045 from vector PTnMOD, bp 1 - 4045

Bp 4051–5694 CMV promoter/enhancer taken from vector pGWIZ (Gene therapy systems), bp 230-1873

Bp 5701-5871 Capsite/Prepro taken from GenBank accession # X07404, bp 563–733

Bp 5878-6012 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site

Bp 6013–6666 Human growth hormone taken from GenBank accession # V00519, bp 1-654

Bp 6673–7080 Conalbumin polyA taken from GenBank accession # Y00407, bp 10651-11058

Bp 7082–10681 from cloning vector pTnMOD, bp 4091-7690

pTnMCS (CHOVep-prepro-ent-hGH-CPA)

Bp 1–3670 from vector PTnMCS, bp 1-3670

- 5 Bp 3676–4350 Chicken Ovalbumin enhancer taken from GenBank accession # S82527.1, bp 1–675

Bp 4357-5692 Chicken Ovalbumin promoter taken from GenBank accession # J00899-M24999, bp 1-1336

Bp 5699–5869 Capsite/Prepro taken from GenBank accession # X07404, bp 563–733

- 10 Bp 5876-6010 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site

Bp 6011–6664 Human growth hormone taken from GenBank accession # V00519, bp 1-654

- 15 Bp 6671–7078 Conalbumin polyA taken from GenBank accession # Y00407, bp 10651-11058

Bp 7080–10679 from cloning vector pTnMCS, bp 3716-7315

pTnMCS (CMV-prepro-ent-hGH-CPA)

Bp 1 – 3670 from vector PTnMCS, bp 1 - 3670

- 20 Bp 3676–5319 CMV promoter/enhancer taken from vector pGWIZ (Gene therapy systems), bp 230-1873

Bp 5326-5496 Capsite/Prepro taken from GenBank accession # X07404, bp 563 – 733

- 25 Bp 5503-5637 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site

Bp 5638–6291 Human growth hormone taken from GenBank accession # V00519, bp 1-654

- Bp 6298–6705 Conalbumin polyA taken from GenBank accession # Y00407, bp 10651-11058

- 30 Bp 6707–10306 from cloning vector pTnMCS, bp 3716-7315

pTnMOD (CHOVep-prepro-ent-hGH-CPA)

Bp 1–4045 from vector PTnMOD, bp 1-4045

- Bp 4051–4725 Chicken Ovalbumin enhancer taken from GenBank accession # S82527.1, bp 1–675
- Bp 4732–6067 Chicken Ovalbumin promoter taken from GenBank accession # J00899-M24999, bp 1-1336
- 5 Bp 6074–6244 Capsite/Prepro taken from GenBank accession # X07404, bp 563–733
- Bp 6251–6385 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site
- Bp 6386–7039 Human growth hormone taken from GenBank accession # V00519, bp 1-654
- 10 Bp 7046–7453 Conalbumin polyA taken from GenBank accession # Y00407, bp 10651-11058
- Bp 7455–11054 from cloning vector pTnMOD, bp 4091-7690

PTnMod(CMV/Transposase/ChickOvep/prepro/ProteinA/ConpolyA)

- 15 BP 1-130 remainder of FI (-) ori of pBluescriptII sk(-) (Stragagene) bp 1-130.
- BP 133-1777 CMV promoter/enhancer taken from vector pGWIZ (Gene Therapy Systems) bp 229-1873.
- BP 1780-2987 Transposase, modified from Tn10 (GenBank #J01829).
- BP 2988-2993 Engineered DOUBLE stop codon.
- 20 BP 2994-3343 non coding DNA from vector pNK2859.
- BP 3344-3386 Lambda DNA from pNK2859.
- BP 3387-3456 70bp of IS10 left from Tn10.
- BP 3457-3674 multiple cloning site from pBluescriptII sk(-) bp 924-707.
- BP 3675-5691 Chicken Ovalbumin enhancer plus promoter from a Topo Clone 10
- 25 maxi 040303 (5' XmaI, 3' BamHI)
- BP 5698-5865 prepro with Cap site amplified from cecropin of pMON200 GenBank # X07404 (5'BamHI, 3'KpnI)
- BP 5872-7338 Protein A gene from GenBank# J01786, mature peptide bp 292-1755 (5'KpnI, 3'SacII)
- 30 BP 7345-7752 ConPolyA from Chicken conalbumin polyA from GenBank # Y00407 bp 10651-11058. (5'SacII, 3'XhoI)
- BP 7753-8195 multiple cloning site from pBluescriptII sk(-) bp 677-235.
- BP 8196-8265 70 bp of IS10 left from Tn10.
- BP 8266-8307 Lamda DNA from pNK2859

BP 8308-9151 noncoding DNA from pNK2859

BP 9152-11352 pBluescriptII sk(-) base vector (Stratagene, INC.) bp 761-2961

#### EXAMPLE 14

##### 5 *Gene Therapy to Treat Cancer in an Animal*

##### Preparation of the transposon-based vector

The following genes were cloned into the transposon-based vector of the present invention: an SV40 promoter linked to the preprosequence of cecropin B together with either a gene of interest encoding Phor14:beta human chorionic gonadotropin (bHCG), a gene of interest encoding gonadotropin releasing hormone (GnRH):Phor 11, or a gene of interest encoding GnRH:Phor 14, each linked to a cecropin B poly A. The Phor peptides are lytic peptides. In this manner, three different vectors were created: 1) pTnPhor14:bHCG; 2) pTnGnRH:Phor 11; and, 3) pTnGnRH:Phor 14. The base vector used was pBTnLac. The SV40 promoter is a constitutive promoter expressed at a moderate level. The cecropin B prepro peptide was selected to permit a peptide to be transported out of the cell. The cecropin B poly A was selected to terminate mRNA synthesis. These vectors in turn stimulate production of the fusion peptides, GnRH:Phor 11 (SEQ IS NO:53), GnRH:Phor 14 (SEQ IS NO:54), and Phor14:bHCG (SEQ IS NO:58). These transposon-based vectors were designed to provide an alternative to conventional chemotherapy in animals with tumors that express a receptor for luteinizing hormone (LH) or GnRH at their surface, for example prostatic, ovarian, breast, pancreatic and some small cell lung carcinomas.

Mice, fish, cats and dogs have received these compositions without any adverse side effects. Temporary sterility was induced in these animals as evidenced by a disrupted reproductive cycle.

The goal of this gene therapy was to administer the transposon-based vector complexed with a transfecting agent to the animal through any desired route (for example intravenous, intraperitoneal, intraarterial, or intramuscular) so that the animal makes the fusion protein. Next, the cells of the animal that expressed a receptor for LH or GnRH recognized and bound the LH or GnRH component of the fusion peptide and delivered the fusion peptide containing the lytic peptide component to the cell, eventually resulting in lysis of the cell.

This approach to gene therapy permits very specific targeting of cells for destruction since cells expressing receptors specific for a ligand are affected without affecting other cells as in conventional chemotherapy or radiotherapy. Further, this approach permits sustained delivery of the fusion peptides over time since the  
5 transgene is stably incorporated.

Use of the transposon-based vector in gene therapy for treatment of cancer in a dog and in a cat

A dog with breast cancer metastasized throughout the body was treated. An  
10 aged female retriever of about 85 pounds body weight was diagnosed with widespread inflammatory mammary carcinoma. The initial diagnosis was confirmed with a skin biopsy of nodular lesions on the left lateral chest wall. The biopsy demonstrated tumor nodules surrounded by fibrous tissue and tumor emboli within dermal lymphatic vessels. The tumor was composed of large, irregular, darkly blue stained  
15 cells with nuclear atypia and a high mitotic rate. The dog was administered the genetic constructs encoding for SEQ ID NO: 54 and SEQ ID NO: 58, i.v., at a dose of 50 ug of each construct in about 150 ul of transfection reagent (Superfect). Ten days later the dog suddenly died. Within three hours, the skin lesion near the original biopsy site was removed and fixed. Histological analysis of sections from this biopsy  
20 revealed advanced and severe necrosis of the tumor cells both within the fibrous lesions and within the lymphatic vessels. Adjacent, normal non-neoplastic cells and tissues were non-necrotic. The death of tumor cells was estimated at more than 90%.

A cat with breast cancer metastasized throughout the body was treated. The cat was diagnosed with widespread inflammatory mammary carcinoma. The initial  
25 diagnosis was confirmed with a skin biopsy of nodular lesions. The cat was administered the genetic constructs encoding for SEQ ID NO: 54 and SEQ ID NO: 58, i.v. at a dose of 50 ug of each construct in about 150 ul of transfection reagent (Superfect). A subsequent histological analysis of sections from this biopsy revealed advanced and severe necrosis of the tumor cells both within the fibrous lesions and  
30 within the lymphatic vessels. Adjacent, normal non-neoplastic cells and tissues were non-necrotic.

The results demonstrate the efficiency of the gene therapy method of the present invention to treat cancer in an animal.

## EXAMPLE 15

*Development of a vector for tissue-specific insulin gene incorporation into animal liver.*

Figure 6 shows a scheme of a pTnMod-based vector for targeting an insulin gene into the liver. Using transposase (ATS) under control of liver-specific promoter, such as liver glucose-6-phosphatase (G6P) promoter, allows for tissue-specific incorporation of the insulin gene in the liver. The insulin gene is also placed under control of a glucose-6 phosphatase (G6P) promoter.

The G6P promoter is cloned from rat genomic liver DNA. Rat genomic liver DNA is prepared according to procedures known to one of ordinary skill in the art. The promoter is cloned by amplifying the gene sequence using specific primers in a PCR reaction using methods known to one of ordinary skill in the art.

Alternatively, rat G6P promoter sequence is deduced from rat G6P gene untranslated upstream region provided in GenBank accession number U57552.1 and a corresponding synthetic oligonucleotide is prepared by methods known to one of ordinary skill in the art, preferably by any one of a number of commercial suppliers of synthetic oligonucleotides.

The gene encoding human proinsulin is amplified from human cDNA according to methods known in the art, for example, by using PCR with the primers specific for a proinsulin gene sequence, which is shown in SEQ ID NO:23. Briefly, total mRNA is isolated from a human pancreas according to procedures standard in the art. This mRNA is used to produce cDNA according to procedures standard in the art. Alternatively, the promoter sequence is amplified by PCR from a commercially available human pancreatic cDNA library, such as the one supplied by Clontech Laboratories, Inc. under 2000 catalog number 7115-1, Human Pancreas QUICK-Clone cDNA.

Proinsulin is PCR-amplified from cDNA using specific primers designed in accordance with the proinsulin DNA sequence. The gene encoding proinsulin is cloned into the multiple cloning site (MCS) of pGWiz downstream of the G6P promoter sequence and upstream of the polyadenylation sequence (polyA).

Each of the identified components is sequenced, and cloned into pTnMod according to the scheme shown in Figure 6. Transposase (ATS) of the pTnMod vector is also placed under the control of the G6P promoter, which is obtained as described

above, by subcloning G6P promoter sequence upstream of the pTnMod transposase sequence. Insertion sequences are denoted IS.

Any other desired components are prepared and incorporated into the vector by methods known to one of ordinary skill in the art.

5           This vector is termed pTnModIns. Sufficient amounts of substantially pure TnModIns DNA are prepared using methods common in the art.

#### EXAMPLE 16

##### *Treatment of rats with the vector for tissue-specific insulin gene incorporation*

10           Diabetic rats are obtained made diabetic by administering the drug streptozotocin (Zanosar; Upjohn, Kalamazoo, MI) at approximately 200 mg/kg.

The rats are bred and maintained according to standard procedures. pTnModIns DNA, an appropriate carrier, and, optionally, a transfection agent, are injected into rats' singhepatic (if using G6P) artery with the purpose of stable  
15 transformation. Incorporation of the insulin gene into the rat genome and levels of insulin expression are ascertained by a variety of methods known in the art. Blood and tissue samples from live or sacrificed animals are tested. A combination of PCR, Southern and Northern blots, in-situ hybridization and related nucleic acid analysis methods are used to determine incorporation of the vector-derived proinsulin DNA  
20 and levels of transcription of the corresponding mRNA in various organs and tissues of the rats. A combination of SDS-PAGE gels, Western Blot analysis, radioimmunoassay, and ELISA and other methods known to one of ordinary skill in the art are used to determine the presence of insulin and the amount produced. Additional transfections of pTnModIns are used to increase protein expression if the  
25 initial amounts of the expressed insulin are not satisfactory, or if the level of expression tapers off. The physiological condition of the rats is closely examined post-transfection to register positive or any negative effects of the gene therapy. Animals are examined over extended periods of time post-transfection in order to monitor the stability of gene incorporation and protein expression.

30           All patents, publications and abstracts cited above are incorporated herein by reference in their entirety. It should be understood that the foregoing relates only to preferred embodiments of the present invention and that numerous modifications or alterations may be made therein without departing from the spirit and the scope of the present invention as defined in the following claims.



## CLAIMS

We Claim:

- 5           1.     A method of providing gene therapy to an animal comprising administering to the animal a transposon-based vector comprising a gene of interest.
- 10           2.     The method of claim 1, wherein the transposon-based vector comprises:
  - 10           a)     a transposase gene operably linked to a first promoter, the transposase gene encoding for a transposase; and
  - b)     one or more genes of interest operably-linked to one or more additional promoters;
  - 15           c)     wherein the one or more genes of interest and their operably-linked promoters are flanked by transposase insertion sequences recognized by the transposase.
- 20           3.     The method of claim 2, wherein the first promoter is a constitutive promoter.
4.     The method of claim 2, wherein the one or more gene of interest is operably-linked to a second promoter.
- 25           5.     The method of claim 1, wherein the gene of interest codes for production of a desired protein, peptide or nucleic acid.

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## GENE THERAPY FOR ANIMALS

### ABSTRACT

Methods and compositions are presented for the administration of transposon-based vectors to animals to provide gene therapy to the animals.

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Attorney Docket No: 51687-0290P (300215)

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FIGURE 1

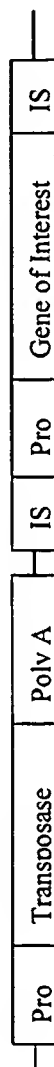


FIGURE 2



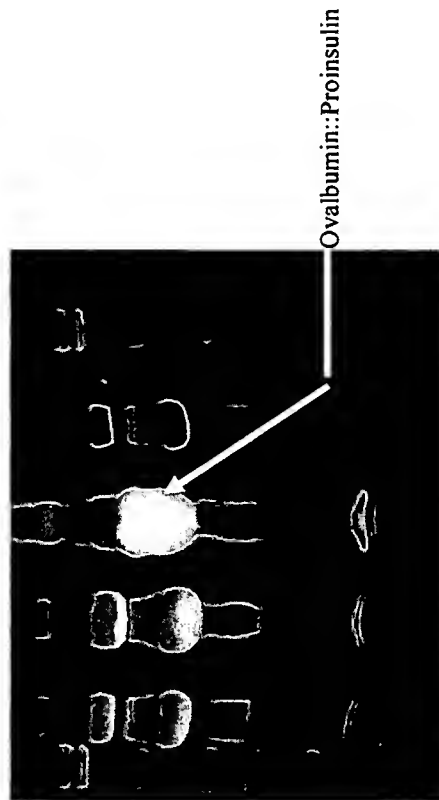
FIGURE 3



FIGURE 4

IS	Tet; Pro	Ovgen	Pro	Ovotrans	Pro	Ovomucin	IS
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FIGURE 5



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Figure 6.

